

H42-A2  
Vol. 27 No. 16  
Replaces H42-A  
Vol. 18 No. 21

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# Enumeration of Immunologically Defined Cell Populations by Flow Cytometry; Approved Guideline—Second Edition

This document provides guidance for the immunophenotypic analysis of non-neoplastic lymphocytes by immunofluorescence-based flow cytometry; sample and instrument quality control; and precautions for acquisition of data from lymphocytes.

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A guideline for global application developed through the Clinical and Laboratory Standards Institute consensus process.



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H42-A2  
ISBN 1-56238-640-9  
ISSN 0273-3099

Volume 27 Number 16

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### Abstract

Clinical and Laboratory Standards Institute document H42-A2—*Enumeration of Immunologically Defined Cell Populations by Flow Cytometry; Approved Guideline—Second Edition* was developed to address issues of procedures and quality assurance for clinical applications of flow cytometry. It is designed to aid clinical laboratorians in the development of quality assurance procedures and to establish the foundation for different laboratories using different commercially available instruments to obtain comparable results. Specific topics covered include: specimen collection, transport, and preparation; sample quality control and staining procedures; instrument calibration; sample analysis; and data analysis, storage, and reporting.

Clinical and Laboratory Standards Institute (CLSI). *Enumeration of Immunologically Defined Cell Populations by Flow Cytometry; Approved Guideline—Second Edition*. CLSI document H42-A2 (ISBN 1-56238-640-9). Clinical and Laboratory Standards Institute, 940 West Valley Road, Suite 1400, Wayne, Pennsylvania 19087-1898 USA, 2007.

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### **Suggested Citation**

(Clinical and Laboratory Standards Institute. *Enumeration of Immunologically Defined Cell Populations by Flow Cytometry; Approved Guideline—Second Edition*. CLSI document H42-A2 [ISBN 1-56238-640-9]. Clinical and Laboratory Standards Institute, 940 West Valley Road, Suite 1400, Wayne, Pennsylvania 19087-1898 USA, 2007.)

### **Proposed Guideline**

December 1989

### **Tentative Guideline**

May 1992

### **Approved Guideline**

December 1998

### **Approved Guideline—Second Edition**

May 2007

ISBN 1-56238-640-9

ISSN 0273-3099

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## Foreword

Advances in the availability and reproducibility of monoclonal antibody reagents specific for a wide range of cell types, coupled with lower costs for increasingly automated flow cytometers with greater data analysis capabilities, have made flow cytometry the method of choice for immunophenotyping hematopoietic cells in the clinical laboratory. CLSI document H42-A2 represents the effort of the CLSI Working Group on Immunophenotyping of Lymphocytes appointed to establish guidelines for enumeration of lymphocyte subsets and CD34<sup>+</sup> hematopoietic stem cells by flow cytometry. In this context, it should be noted that for both types of assays, similar guidelines already have been developed by specific professional organizations or at national levels.<sup>1-10</sup> The current guideline aims to bring the state-of-the-art techniques together in a comprehensive, but readily usable format. It should be recognized that on occasion, national guidelines will override this document where applicable.

H42-A2 is designed to aid clinical laboratorians in the development of quality assurance procedures and to establish the foundation for laboratories using different commercially available instruments to obtain comparable results. This document should help minimize interoperator and interlaboratory variability in the various components of flow cytometry. Specific topics covered include specimen collection, transport, and preparation; sample quality control and staining procedures; instrument calibration; sample analysis; and data analysis, storage, and reporting.

In an effort to create an easy-to-use guideline, the main body of the H42-A2 document was divided into three parts. Part A: *General* includes the Scope, Introduction, Standard Precautions, Overview, Definitions, Safety, and an introductory section on methods for enumeration of absolute cell numbers. Part B: *Enumeration of Lymphocyte Subsets* includes recommendations related to the collection, transport, preparation, and analysis of specimens for enumeration of lymphocyte subsets. Part C: *Enumeration of CD34<sup>+</sup> Hematopoietic Stem and Progenitor Cells*, includes specific recommendations for the enumeration of CD34<sup>+</sup> stem cells, as well as—for convenience—some of the same general information provided in Part B (e.g., patient information, venipuncture technique, labeling of specimen).

## A Note on Terminology

CLSI, as a global leader in standardization, is firmly committed to achieving global harmonization wherever possible. Harmonization is a process of recognizing, understanding, and explaining differences while taking steps to achieve worldwide uniformity. CLSI recognizes that medical conventions in the global metrological community have evolved differently in the United States, Europe, and elsewhere; that these differences are reflected in CLSI, ISO, and CEN documents; and that legally required use of terms, regional usage, and different consensus timelines are all obstacles to harmonization. In light of this, CLSI recognizes that harmonization of terms facilitates the global application of standards and deserves immediate attention. Implementation of this policy must be an evolutionary and educational process that begins with new projects and revisions of existing documents.

In order to align the usage of terminology in this document with that of ISO, the term *accuracy*, in its metrological sense, refers to the closeness of the agreement between the result of a (single) measurement and a true value of a measurand, and comprises both random and systematic effects.

The term *diagnostic sensitivity* is combined with the term *clinical sensitivity*, and correspondingly the term *diagnostic specificity* is combined with the term *clinical specificity*, because in Europe, the term “clinical” often refers to clinical studies of drugs under stringent conditions.

All terms and definitions will be reviewed again for consistency with international use, and revised appropriately during the next scheduled revision of this document.

**Key Words**

Autofluorescence, CD system, CD34<sup>+</sup> hematopoietic stem cells, color compensation, dual-parameter display, flow cytometry, fluorescein isothiocyanate (FITC), fluorescence, forward angle light scatter, gate, histogram, immunoglobulin, immunophenotyping, linear amplification, logarithmic amplification, low angle light scatter, lymphocyte, lymphocyte subsets, 90° light scatter, phycoerythrin (PE), positive procedure control, single-parameter display (histogram), subclass

# **Enumeration of Immunologically Defined Cell Populations by Flow Cytometry; Approved Guideline—Second Edition**

## **PART A: General**

### **1 Scope**

The scope of this document is to establish performance guidelines for the identification and enumeration of lymphocyte subpopulations and the enumeration of CD34<sup>+</sup> hematopoietic progenitors using immunofluorescence-based flow cytometry (FCM).

The working group recognizes that other, so-called nontraditional methodologies exist or are in development for enumeration of CD4<sup>+</sup> T-lymphocytes (e.g., systems using microcapillary sample delivery or nonfluorescent cell detection; see Appendix C). Some of the issues discussed in this document are common to the use of any method for CD4<sup>+</sup> T-cell enumeration (e.g., sample collection and transport, safety issues, data reporting, and interpretation). However, issues such as sample preparation, instrument calibration, and quality control differ significantly for nontraditional methodologies and are not discussed in this document.

Presently, there are no universally accepted standards for precision, accuracy, and interlaboratory comparability in lymphocyte enumeration by FCM. General consensus was reached on the basic International Society for Hematotherapy and Graft Engineering (ISHAGE)<sup>9</sup> guidelines for CD34 analysis, and this forms the basis of the technique described herein. It is beyond the scope of this document to establish general performance criteria and reference intervals. Therefore, it is each laboratory's responsibility to establish instrument performance criteria and staining characteristics for its own specific reagents.

### **2 Introduction**

Flow cytometry is an established technology that has moved from the research laboratory into the clinical laboratory. The goal of this document is to establish quality assurance procedures that will help ensure precision and accuracy of flow cytometric results appropriate for their use in the clinical laboratory. Since at present, most assays for lymphocyte subset and hematopoietic progenitor enumeration in clinical laboratories are fluorescence-based, this document is limited to specific issues surrounding the use of such systems. Major points of attention include the following:

- potentially biohazardous procedures and appropriate precautions;
- type and frequency of methodologic controls required;
- analysis methods for lymphocyte subset and hematopoietic progenitor identification;
- methods for determination of absolute cell concentrations;
- guidelines for retention of laboratory records; and
- guidelines for definition of laboratory reference intervals.

### 3 Standard Precautions

Because it is often impossible to know what isolates or specimens might be infectious, all patient and laboratory specimens are treated as infectious and handled according to “standard precautions.” Standard precautions are guidelines that combine the major features of “universal precautions and body substance isolation” practices. Standard precautions cover the transmission of all infectious agents and thus are more comprehensive than universal precautions, which are intended to apply only to transmission of blood-borne pathogens. Standard and universal precaution guidelines are available from the US Centers for Disease Control and Prevention (Garner JS, Hospital Infection Control Practices Advisory Committee. Guideline for isolation precautions in hospitals. *Infect Control Hosp Epidemiol.* 1996;17(1):53-80). For specific precautions for preventing the laboratory transmission of all infectious agents from laboratory instruments and materials and for recommendations for the management of exposure to all infectious disease, refer to the most current edition of CLSI document M29—*Protection of Laboratory Workers From Occupationally Acquired Infections*.

### 4 Overview

In a previous document, the original subcommittee outlined general principles that are useful for performing FCM on blood samples for lymphocyte subset analysis. Some of these principles are directly applicable to the enumeration of CD34<sup>+</sup> hematopoietic stem and progenitor cells. Because the latter analysis involves rare event detection, special procedures not essential for lymphocyte subset enumeration are necessary. This section outlines the most important differences; detailed information is then presented in appropriate sections to follow.

#### 4.1 Goals

The principal goal of blood lymphocyte immunophenotyping is to enumerate antigenically defined lymphocyte subsets. T-cells are the main lymphocyte subset and are defined by their expression of CD3. The major subsets of T-cells are the so-called T-helper cells (CD3<sup>+</sup> and CD4<sup>+</sup>) and the cytotoxic T-cells (CD3<sup>+</sup> and CD8<sup>+</sup>). Smaller lymphocyte subsets include the B-cells (CD19<sup>+</sup>) and NK cells (CD3<sup>-</sup> and CD56<sup>+</sup>).

CD34<sup>+</sup> cell enumeration is used in the evaluation of patients undergoing hematopoietic stem cell and progenitor cell transplants. CD34 is an antigen expressed on hematopoietic stem and progenitor cells.

#### 4.2 Quality Control Procedures

Instrument setup for CD34<sup>+</sup> cell enumeration is generally similar to that used for lymphocyte enumeration. The use of the viability dye 7-aminoactinomycin D (7-AAD) necessitates accurate compensation using this dye, and this point is addressed in the instrument setup section (see Appendix A).

#### 4.3 Sample Preparation

Although the methods initially developed for processing blood may be applicable to the study of bone marrow, cord blood, apheresis, or other types of samples, special care is often necessary to ensure that the cell concentration is appropriate for the amounts of antibody used, and methods for RBC lysis do not impact on the viability of cells of interest.

#### 4.4 Reagents

The reagent panels used for lymphocyte subset enumeration are fairly well-standardized and usually include CD45 and/or CD3 as a gating reagent. CD4 and CD8 subsets are routinely reported as a subset of

CD3<sup>+</sup> cells, allowing exclusion of other populations that may react with these antibodies (CD4<sup>+</sup> monocytes, CD8<sup>+</sup> NK cells). Appendix B outlines a method for CD4 enumeration based on CD45 and side scatter analysis, and may economize on reagent costs.<sup>11</sup> Antibodies to CD34 are present in three main classes. In general, Class III antibodies are recommended as they react with CD34<sup>+</sup> cells from all specimen types, irrespective of the type of fluorochrome with which they have been conjugated.

## 4.5 Sample Analysis

While lymphocyte subsets can usually be enumerated accurately with as little as a few thousand CD3<sup>+</sup> events, CD34<sup>+</sup> cells may only represent a very small portion of the cells analyzed, and large numbers of events must be acquired to gather sufficient data on the cells of interest. Due to technical advances, management of large data files is currently less cumbersome, and it is recommended that all available information is acquired (i.e., acquisition in “list mode”). An exclusion gate that removes CD45<sup>+</sup>, CD34<sup>+</sup> events is helpful in reducing overall file size, particularly in blood samples with incomplete RBC lysis, cord blood, or bone marrow samples. Because the data that are thus collected may be complex and contain several different populations, many different gates may have to be set later when the data are analyzed.

## 4.6 Data Analysis and Interpretation

### 4.6.1 Data Analysis

For lymphocyte subset analysis, results are classically given in both percentages and absolute counts. For CD34 analysis, absolute counts are increasingly used. However, the conventional method of analyzing histograms—in which a cursor is placed at the boundary of a negative control and events in the test histogram to the right of this boundary are considered positive—is not appropriate for analyzing CD34<sup>+</sup> cell distributions because control histograms often show considerable overlap with test histograms containing low-intensity positive cells. Fluorescence intensity is an important variable in CD34 analysis and the analysis of this rare cell population relies heavily on this characteristic.

### 4.6.2 Data Interpretation

Results from FCM studies should be reported to give the composite lymphocyte immunophenotype in both percent and absolute numbers. A CD4/CD8 ratio may provide additional helpful information. For CD34 analysis, absolute CD34 cells present should be reported. Additionally, for samples collected from apheresis procedures, it is important that results are expressed as number of CD34<sup>+</sup> cells available per kilogram of body weight of the patient. This calculation may be done within the FCM laboratory or the transplant laboratory. Coordination and validation of this final calculation is essential to ensure that calculation errors are avoided at this stage.

## 5 Terminology

### 5.1 Definitions

**allophycocyanin//APC** – one of several phycobiliprotein-based fluorochromes, derived from algae or bacteria, which can be conjugated to antibodies for use in immunophenotyping; **NOTE:** APC has a molecular weight of roughly 80 000 daltons. Although optimally excited at 655 nm, there is sufficient excitation at 635 nm to produce a useable signal at 660 nm, when excited with a helium-neon (HeNe) or diode laser.

**analysis boundary** – dividing line (cursor) placed on a histogram or dual-parameter display that discriminates events that are considered positive or reactive with a particular antibody from those that are

negative or nonreactive; **NOTE:** An analysis boundary is most commonly set on a histogram obtained using a negative control antibody so that a fixed small proportion of events is considered positive.

**autofluorescence** – the intrinsic fluorescence of unstained cells, generally caused by pyrimidines and flavin nucleotides; **NOTE:** The level of autofluorescence is a function of the excitation wavelength and varies with the cell type analyzed and/or the state of cellular activation. Cultured cell lines, neutrophils, and macrophages usually demonstrate higher levels of autofluorescence with 488 nm excitation, and proportional, lower autofluorescence with excitation at longer wavelengths (e.g., 635 nm). Autofluorescence can be decreased by specific sample preparation procedures (e.g., incubation with crystal violet).

**cluster of differentiation (CD) system** – the identification of monoclonal antibodies with similar patterns of reactivity with human cells was the focus of numerous international workshops; **NOTE:** Each group of antibodies was assigned a CD number. Not all antibodies in a CD group react with identical portions (epitopes) of their target antigen. An antigen recognized by a given cluster of antibodies (e.g., CD4) is referred to as a “CD antigen” (e.g., CD4 antigen). An antibody that belongs to a given cluster is referred to as CD “x,” with the manufacturer’s nomenclature given in parentheses (e.g., CD4 [Leu3a]).

**color compensation** – a mathematical or electronic correction applied to flow cytometric data to account for the overlapping fluorescence emission from one fluorochrome into the wavelength region where the second is measured; **NOTE:** Compensation is achieved by subtracting a fraction of one measured fluorescent signal from each of the other fluorescent signals, the amount of subtraction determined from appropriate single-stained controls. The number of signals involved is equal to the number of fluorochromes (N) used and the number of compensation coefficients to be determined represented by an N x N dimensional matrix. The result is that each corrected signal reflects the emission of a single fluorochrome.

**counting beads** – fluorescent microspheres of known concentration, which are combined in a known amount with a sample in order to assess the concentration of a population in that sample (e.g., lymphocyte subsets, CD34<sup>+</sup> hematopoietic stem cells).

**dual-parameter display//dot plot//contour plot//two-parameter histogram** – graphical representation of data in which correlated values for two different parameters measured on the same cell are plotted on an x,y grid.

**dual-platform method** – a method for the determination of absolute cell concentration using data derived both from flow cytometric measurements and a second instrument, generally a hematology analyzer; **NOTE:** The flow cytometer is used to obtain the fraction of the cellular subpopulation of interest present within a more abundant parent cell population, usually either total lymphocytes or total WBC. The absolute concentration of the parent population is provided by an independent assay of the sample on a second instrument. The product of these two measurements gives the absolute concentration of the population of interest. The disadvantage of the method is a compounding of the error inherent in each of the two measurements, and for this reason is judged inferior to single-platform methodologies.

**epitope//antigenic determinant** – that portion of an antigen against which the specific binding region of a monoclonal antibody reagent is directed; **NOTE:** Epitopes may be linear sequences of as few as six amino acids or conformationally determined sections of the antigen. Each antigen typically contains multiple epitopes.

**event** – a single particle or cell identified by a flow cytometer.

**external quality assessment** – evaluation of the laboratory’s performance on examination of samples of external origin for the purposes of determining adequacy of the laboratory’s preexamination, examination,

and postexamination activities (ISO/IEC Guide 43-1)<sup>12</sup>; **NOTE:** The main object is to establish between-laboratory and between-instrument comparability that is, if possible, in agreement with a reference standard (where one exists). External quality assessment schemes may be regional, national, or international. They may also be limited to the users of a particular instrument. It is sometimes also referred to as “proficiency testing,” especially when the external agency is a regulatory agency.

**fluorescein isothiocyanate//FITC** – the most common fluorochrome used for cell immunophenotyping; **NOTE:** Fluorescein conjugates absorb maximally at approximately 490 nm, close to the 488 nm emission of argon lasers, and emit maximally near 525 nm. Each conjugated fluorescein molecule adds a net negative charge to the antibody and therefore, may change its potential binding characteristics.

**fluorescence intensity** – a measurement of the amount of fluorochrome bound to a particle or cell; **NOTE:** Increasing intensity is reflected in a fluorescence signal appearing in a higher channel number. Under appropriate conditions, fluorescence intensity can be related to the number of binding sites a cell has for a particular fluorochrome-conjugated reagent.

**forward angle light scatter//low angle//FALS//FSC//FS** – measurement of light at a low radial angle relative to the incident light source; **NOTE:** Measured values are a function of the cross-sectional area and refractive index of a cell or particle and the wavelength used for measurement. It is commonly used as an estimate of the relative size of a cell or particle.

**gate** – a set of parameters used to electronically select particular cells for evaluation; **NOTE:** Typically, a region of interest is defined based on one set of parameters (such as FSC vs. SSC), and other parameters (such as fluorescence) are evaluated only for cells within that defined region.

**immunophenotyping** – characterization of cells by the identification of cell surface or intracellular antigens, utilizing antibody reagents that recognize specific cell-associated molecules; **NOTE:** In this guideline, the cells that are immunophenotyped are lymphocytes and hematopoietic stem and progenitor cells.

**internal control** – cells of known phenotypic expression present in the sample analyzed; **NOTE:** Positive and/or negative expression and fluorescent intensity of such cells serve as process controls.

**irreplaceable specimen** – a specimen that is difficult to collect (invasive and/or surgically removed) and cannot be replaced by a repeat specimen, such as a lymph node or spleen; **NOTE:** Such specimens may be analyzed even when there is poor viability, since another identical diagnostic specimen cannot be obtained. This is in contrast to blood, which can be easily replaced with minimal impact on the patient’s health. There are instances in which a blood sample may be considered irreplaceable, such as when recollection may delay diagnosis of an acute leukemia, or therapy may already have been initiated based on presumptive clinical diagnosis.

**linear amplification** – a linear amplifier (amp) produces a signal output directly proportional to the input signal amplitude; **NOTE:** For example, a linear amp could have output varying from 1 to 5 V as the input signal varies from 0.01 to 0.05 V.

**list mode** – a data storage format in which individual values of all parameters for each cell in a sample are retained, allowing maximum flexibility for subsequent analysis.

**live gate** – also called an acquisition gate or enrichment gating, this represents a set of parameters used to collect data on a population of cells for further analysis; **NOTE:** A live gate is typically set when the population of interest represents only a minor fraction of the total population, but when one or more parameters clearly distinguishes this population from the larger population of less important cells.

**logarithmic amplification** – a logarithmic amplifier (log amp) produces an output signal value proportional to the logarithm of the input signal value; **NOTE:** A four-decade log amp might, for example, have an output of 0 V for a 1-mV input; 2.5 V for a 10-mV input; 5 V for a 100-mV input; 7.5 V for a 1-V input; and 10 V for a 10-V input. Log amps are useful when analyzing samples containing cells whose measured parameters differ by orders of magnitude.

**lysing agent** – a reagent used to remove or lyse RBC from a sample in order to simplify the analysis of WBC; **NOTE:** Ammonium chloride in a bicarbonate buffer is the most commonly used lysing agent, although a variety of commercial preparations are also available.

**peridynyl chlorophyllin/PerCP** – a fluorochrome derived from the photosynthetic apparatus found in the dinoflagellate, *Glenodinium*; **NOTE:** PerCP is a protein complex with a molecular weight of approximately 35 kDa. PerCP has an excitation maximum around 490 nm, with an emission spectrum that peaks at 675 nm. Due to its photobleaching characteristics, PerCP conjugates are not recommended for use on stream-in-air flow cytometers and have been replaced in many applications by the tandem fluorochrome PerCP-Cy5.5 that is both brighter and has improved photostability.

**phycoerythrin/PE** – one of several phycobiliprotein-based fluorochromes, derived from algae or bacteria, which can be conjugated to antibodies for use in immunophenotyping; **NOTE 1:** PE has a molecular weight of 240 000 kD. Although it absorbs light maximally at 545 nm, there is sufficient absorption at the 488 nm excitation to provide a usable signal in the red/orange (575 nm) emission range when excited by an argon laser; **NOTE 2:** Denaturation of PE does not cause a spectral shift but instead quenches the fluorescence. Aggregation of PE/immunoglobulin conjugates causes nonspecific binding.

**primary antibody** – an antibody that binds directly to the specific protein of interest.

**procedure control** – a specimen that is drawn, prepared, and stained with the same protocol used for patient specimens; **NOTE:** It is used: (1) for instrument setup and monitoring to establish test-specific instrument settings and to establish color compensation settings; and (2) as a control specimen for sample and data analysis.

**quality assurance** – part of quality management focused on providing confidence that quality requirements will be fulfilled (ISO 9000)<sup>13</sup>; **NOTE:** The practice that encompasses all procedures and activities directed toward ensuring that a specified quality of product is achieved and maintained. In the testing environment, this includes monitoring all the raw materials, supplies, instruments, procedures, sample collection/transport/storage/processing, recordkeeping, calibrating and maintenance of equipment, QC, proficiency testing, training of personnel, and all else involved in the production of the data reported.

**quality control (QC) (internal)** – the set of procedures undertaken in a laboratory for the continual assessment of work carried out within the laboratory and evaluation of the results of tests to decide whether the latter are reliable enough for release to the requesting clinician; **NOTE:** The procedures should include tests on control material and statistical analysis of patient data. The main object is to ensure day-to-day consistency of measurement or observation that is, if possible, in agreement with an agreed reference, such as control material with assigned values.

**rare event analysis** – analysis of cells present at low frequencies, such as stem cell enumeration or detection of minimal residual disease.

**reference interval** – the range of values obtained on specimens from normal controls assayed under the conditions used for patient specimens; **NOTE:** Assayed values that fall outside the reference interval are considered abnormal and may indicate a disease state. Relevant reference intervals are dependent on the assay methodology employed and on the composition of the patient population studied (e.g., with respect to age and sex).

**resolution** – the ability to discriminate between cells or particles having different signal intensities; **NOTE:** The ability to resolve discrete populations is a function of biological factors (heterogeneity of signal within each population, difference in signal intensities between populations), as well as instrument factors (sensitivity, variation of measurement [CV]).

**reverse pipetting** – technique recommended for pipetting solutions with a high viscosity (such as blood) or those which tend to foam; **NOTE:** The advantage is reduction of errors due to fluid film retention. Reverse pipetting is only possible with air displacement pipettes. The sequence of operations is reversed as compared to standard pipetting: more fluid is initially aspirated than will be dispensed, and the remainder is discarded (see Section 7.5.2.3 for a detailed description).

**sample (patient)** – one or more parts taken from a system, and intended to provide information on the system, often to serve as a basis for decision on the system or its production (ISO 15189);<sup>14</sup> **NOTE 1:** For example, a volume of serum taken from a larger volume of serum (ISO 15189); **NOTE 2:** A sample is prepared from the patient specimen and used to obtain information by means of a specific laboratory test.

**secondary antibody** – an antibody that is labeled by fluorescent tag or biotin and binds to a primary antibody to allow its detection.

**sensitivity** – **1)** in this document, the ability to distinguish signals of interest from background optical or electronic “noise” or biological signals, such as those due to autofluorescence or nonspecific reagent binding; **2)** the change in response of a measuring system or instrument divided by the corresponding change in the stimulus (modified from VIM93).<sup>15</sup>

**side scatter (right angle [90° LS], wide-angle, side scatter [SSC], or orthogonal light scatter)** – measurement of light at right angles to the incident light source; **NOTE:** This measurement is related to the internal and surface complexity of a cell or particle, such as cytoplasmic granularity, membrane irregularity, and/or nuclear shape.

**single-parameter display/histogram** – frequency distribution of measured signal intensities (i.e., channel numbers) observed for cells within a population.

**single-platform method** – a method for the determination of absolute cell concentration with all data derived from flow cytometric measurements; **NOTE:** The determination of concentration requires an assessment of the volume of sample analyzed, and may be determined either directly by volumetric methods or indirectly by the addition of fluorescent beads at a known concentration. The latter method is more commonly used with the current generation of instrumentation. Single-platform methods are judged superior to dual-platform methods (see **dual-platform method** above).

**specimen (patient)** – the discrete portion of a body fluid or tissue taken for examination, study, or analysis of one or more quantities or characteristics, to determine the character of the whole; **NOTE:** The substance may still be referred to as a specimen if it has been processed from the obtained specimen; thus, examples of specimens include whole blood and serum or plasma prepared from whole blood; saliva; CSF; feces; urine; fingernail clippings; hair clippings; tissue samples, even if embedded in a paraffin block; etc.

**subclass control** – an immunoglobulin of the same isotype (class or subclass) as the monoclonal antibody of interest, but without specificity for any known human antigens; **NOTE:** Most monoclonal antibodies used in FCM are either IgM, or various subclasses of IgG (IgG1, IgG2a, IgG2b, or IgG3). This type of control is typically used to estimate nonspecific binding for antibody reagents of a given subclass.

**tandem conjugate** – a chemical complex of two fluorochromes arranged so that some of the energy emitted from the first is used to excite the second; **NOTE:** In practice, the most common tandem

conjugates link phycoerythrin (PE) or other fluorochromes, which can be excited at 488 nm, to a fluorochrome that normally is excited at a longer wavelength than 488 nm, and that emits in the far red part of the spectrum. Examples of tandem conjugates are PE-Cy5, PE-Cy7, and APC-Cy7.

**threshold** – the level of signal above which a measured value is considered significantly different than background “noise”; **NOTE:** Threshold for instrument fluorescence sensitivity is the level of signal found for what is considered a nonfluorescent object. Threshold for determination of positive antibody staining is the level of signal found for cells not believed to react specifically with a given antibody reagent.

**viability dye** – a dye that may serve to identify nonviable cells by virtue of its ability to penetrate cells with damaged membranes and subsequently bind to double-stranded nucleic acid; **NOTE:** Propidium iodide (PI) and 7-aminoactinomycin D (7-AAD) are the most commonly used viability dyes. These viability dyes cannot be used as an indication of viability after fixation of cells by most methods because all cells become permeable and take up PI.

**whole blood lysis** – a method of specimen preparation where antibody reagents are added to an aliquot of whole blood and, following incubation, a lysing agent added to remove RBC; **NOTE:** A subsequent wash of the sample may be used to reduce background fluorescence, but should not be performed if absolute counting beads are added. The primary advantage of the method is that it allows interaction of the antibody reagent with cells in a near native state and minimizes sample handling.

## 5.2 Acronyms and Abbreviations

|        |                                                               |
|--------|---------------------------------------------------------------|
| 7-AAD  | 7-aminoactinomycin D                                          |
| ACD    | acid citrate dextrose                                         |
| amp    | amplifier                                                     |
| APC    | allophycocyanate                                              |
| BSC    | biological safety cabinet                                     |
| BSL-2  | Biosafety Level 2                                             |
| CSF    | cerebrospinal fluid                                           |
| CV     | coefficient of variation                                      |
| DP     | dual platform                                                 |
| EDTA   | ethylene-diaminetetraacetic acid                              |
| FCM    | flow cytometry                                                |
| FITC   | fluorescein isothiocyanate                                    |
| FSC    | forward angle light scatter                                   |
| HPC    | hematopoietic progenitor cells                                |
| HPC-A  | hematopoietic progenitor cells – apheresis                    |
| HPC-C  | hematopoietic progenitor cells – cord blood                   |
| HPC-M  | hematopoietic progenitor cells – marrow                       |
| IC     | isotope control                                               |
| ISHAGE | International Society for Hematotherapy and Graft Engineering |
| kD     | kilodalton                                                    |
| LS     | light scatter                                                 |
| mAb    | monoclonal antibody                                           |
| MCF    | mean channel fluorescence                                     |
| mV     | millivolt                                                     |
| PE     | phycoerythrin                                                 |
| PerCP  | peridiny chlorophyllin                                        |
| PI     | propidium iodide                                              |
| QFCM   | quantitative flow cytometry                                   |
| RBC    | red blood cell(s), erythrocyte(s)                             |
| SP     | single platform                                               |

|     |                                   |
|-----|-----------------------------------|
| SSC | side scatter                      |
| V   | volt                              |
| WBC | white blood cell(s), leukocyte(s) |
| WHO | World Health Organization         |

## **6 Safety**

There are many infectious agents that may be present in blood and body fluids. Because the sample may contain the active agent of infection with resulting morbidity or mortality, all specimens should be handled as though they are potentially infectious. See also Section 3 (Standard Precautions). For more information, refer to the most current editions of CLSI/NCCLS documents H3—*Procedures for the Collection of Diagnostic Blood Specimens by Venipuncture*, and M29—*Protection of Laboratory Workers From Occupationally Acquired Infections*.

### **6.1 Specimen Collection**

All human body fluids and tissues should be treated as potentially infectious. Blood should be collected as outlined in CLSI document M29. Care should be taken to prevent needlestick injuries. Needles should be discarded in a puncture-resistant container and they should not be resheathed, purposefully bent, cut, or removed from the syringe.

### **6.2 Safety Attire**

Gloves and laboratory coats should be worn by all personnel processing blood specimens. If splashing or spattering of the specimen is anticipated, masks and protective eyewear should be worn to prevent exposure of eyes, nose, and mouth. Gloves should be changed and hands washed after completion of the work and before leaving the laboratory. For more detailed information, see the most current edition of CLSI document M29.

### **6.3 Biological Safety Cabinets**

If possible, all work should be done in a biological safety cabinet (BSC, Class I or II). If this is not possible, all procedures that have the potential for creating droplets or aerosols (vortexing, opening evacuated tubes, etc.) should be performed in a BSC (Class I or II).

### **6.4 Specimen Containers**

All specimens should be aliquoted into tubes that have caps or microtiter plates that have covers (preferably sealed covers). The caps and covers should be in place except when adding to or removing from the containers.

### **6.5 Centrifugation**

If specimens are centrifuged, care should be taken to avoid aerosol formation (e.g., in sealed vessels or inside safety carriers).

### **6.6 Pipetting**

Pipetting by mouth is not allowed. Manual or electronic pipetting devices must be used.

## 6.7 Sharp Devices

Use of sharp devices (e.g., needles, glass Pasteur pipettes, glass containers) should be avoided as much as possible.

## 6.8 Blood Spills

If a blood spill occurs, it should be decontaminated at once with an appropriate agent, such as 1/10 volume dilution of freshly (daily) prepared 0.71 mol/L sodium hypochlorite (i.e., undiluted household bleach) or appropriately diluted mycobactericidal hospital disinfectants. See the most current edition of CLSI/NCCLS document GP17—*Clinical Laboratory Safety* for additional information.

## 6.9 Waste Disposal and Specimen Inactivation

Collect used specimens and all disposable laboratory supplies, including needles and sharp elements, in puncture-resistant containers designed specifically for such materials. Dispose of by appropriate means (e.g., incineration or autoclaving), in conformity with local regulations.

Chemical techniques for specimen inactivation include mixing samples with a broad spectrum iodophor or bleach for 24 hours, followed by proper disposal. Bleach cannot be used with ammonium chloride-containing solutions, as dangerous chlorine gas will be released.

For more information on the handling and disposal of medical waste, refer to the most current edition of CLSI/NCCLS document GP5—*Clinical Laboratory Waste Management*.

## 6.10 Specimen Storage

It is preferable to analyze only samples in which infectious agents have been inactivated. Commercially available combined lysing and fixing solutions have been shown to inactivate viral agents like HIV by three to five logs, while ammonium chloride alone had little effect.<sup>16</sup> If samples have not been lysed using a virus-inactivating lysing reagent, they should be resuspended in a solution of buffered (pH 7.0 to 7.4) paraformaldehyde or formaldehyde (commonly 0.1 to 2.0%) after the last centrifugation step in the labeling procedure, and stored at 4 °C until analysis. The minimum storage time in (para)formaldehyde required to inactivate potentially infectious agents is not clearly established. Fixation is also used for stabilization of the sample so that it may be analyzed at a later time. Because specimen preparation and fixation techniques may vary between laboratories, the concentration of (para)formaldehyde and maximum storage time should be verified by each individual laboratory.

## 6.11 Unfixed Specimens

When unfixed specimens are analyzed on the flow cytometer, the laboratory must be able to demonstrate that appropriate biosafety procedures are in place to minimize exposure to infectious material. Because aerosol generation in flow cytometers with a stream-in-air is a potential hazard, special attention must be exercised to operate such an instrument in accordance with the manufacturer's biosafety recommendations.

## 6.12 Equipment Disinfection

The waste container on the flow cytometer should contain 1/10 of its volume of 0.71 mol/L sodium hypochlorite (undiluted household bleach).

Laboratory equipment should be properly disinfected with a solution known to inactivate infectious agents or freshly made 10% solution of household bleach. It is particularly important that instruments be properly disinfected before repair or maintenance is performed (see the most current edition of CLSI/NCCLS document GP17—*Clinical Laboratory Safety*).

## 7 Methods for Enumeration of Absolute Cell Numbers

### 7.1 Introduction and Historical Perspective

In the early 1980s, FCM became the tool of choice for assessing immune status of individuals suspected of suffering from acquired immunodeficiency syndrome (AIDS).<sup>17</sup> Blood specimens were spun down in density gradient tubes to isolate WBC for immunophenotyping. This type of cell processing was compatible with the open hydrodynamic flow cells on most research cytometers at that time. The emphasis was on acquiring a sufficient number of cells to obtain statistically robust data from a specimen, rather than restricting the analysis to a set volume of specimen. First, the possibility and then the clinical demand for enumerating T-cell subset concentrations were followed by the introduction of the “lyse-no-wash” whole blood staining method using RBC lysis.<sup>18</sup> Unfortunately, most bench-top flow cytometers for clinical use are manufactured to this day without a volumetric sample delivery system.

The absolute cell concentration is defined as the number of cells of interest in a known volume of specimen. By their design, flow cytometers are able to easily enumerate cells, but as direct volumetric measurement is not implemented on most current instruments, indirect methods must be employed to determine the volume of specimen assayed. The “dual-platform” (DP) method (see Section 7.2 below) relies on measurements from two instruments, typically a hematology analyzer and a flow cytometer, resulting in a compounding of the error present in both methodologies. In contrast, the “single-platform” (SP) method (see Section 7.3 below) obtains all measurements from the flow cytometer, but requires accurate pipetting of blood and reagents. By comparison, the single-platform method has been demonstrated to have improved precision over the dual-platform method with resulting reduction of interlaboratory variability.<sup>19,20</sup> Therefore, this document will focus on SP-based methods for the enumeration of lymphocyte subset and CD34 absolute cell concentrations.

### 7.2 Dual-Platform (DP) Methods

Initially, absolute T-cell subset counts were derived from three measurements determined with two instruments: the absolute WBC count, the percentage of WBC that are lymphocytes (differential count; both determined by a hematology analyzer), and the percentage of lymphocytes that are CD4<sup>+</sup> T-cells (determined by FCM). Later, the “PanLeucogating” approach was developed, in which the absolute CD4<sup>+</sup> T-cell count was derived from the absolute WBC count and the percent CD4<sup>+</sup> T-cells expressed as proportion of WBC (described in detail in Appendix B).<sup>11</sup> This approach reduces the variability of this assay by avoiding the percent lymphocytes as determined by the hematology analyzer, which is relatively inaccurate. By the same token, the original ISHAGE guidelines for CD34 enumeration<sup>9</sup> were based on DP technology; later, an SP version was published.<sup>5</sup> With the recognition of the clear advantages of SP methods and the increasing need for simultaneous viability measurements, DP methods have been superseded by SP methods that are summarized in Section 7.3, and described in more detail in Part B.<sup>21</sup>

### 7.3 Single-Platform (SP) Methods Based on Volumetry

As long as lymphocytes were separated from WBC by density gradients and washed before immunophenotyping, T-cell subset results were reported as percentage of lymphocytes. Once sample preparation for immunophenotyping evolved to the direct use of whole blood specimens (i.e., “lyse, stain, no-wash” methods), the demand for absolute CD4<sup>+</sup> T-cell counts increased. Theoretically, modification of instruments for clinical use to include volumetric sample delivery would be a simple task, but this was a significant challenge for instrument manufacturers. The two major manufacturers of flow cytometers for

clinical use have opted for a reagent-based add-on solution that requires additional software to calculate unit volume blood using existing instrument fluid propulsion. This technology is based on the use of microfluorospheres for flow rate monitoring during the sample analysis phase of immunophenotyping (see Section 7.4). A few manufacturers have integrated a volume delivery module into the instrument fluidics system. There are two types of integrated volumetric delivery systems available for SP methods. The traditional method was a step-motor driven precision syringe pump system. Less commonly, a dual linear, air-liquid interface-sensing, start/stop device with a syringe system is also available. In either case, on-board software takes care of any required calculations to adjust between the actual measured volume observed in the optical path, and the volume ( $\mu\text{L}$ ) of whole blood. Only recently, both solutions appeared on the lower-priced instruments. However, there is still a lack of sufficient evidence to support reliability claims for these volumetric technologies. It is important to note that not all volumetric instruments provide the reporting format that is required for pediatric patients infected with HIV (i.e., T-cell subset results expressed as percentage of lymphocytes).

## 7.4 SP Methods Based on Counting Beads

This method is designed to enable determinations of both absolute and percentage lymphocyte subset values, or absolute CD34 counts using a single tube. The methods are based on the addition of a known number of fluorescent microspheres to a known volume of sample, either using a tube containing a pellet consisting of a known number of fluorescent microspheres to which sample is added or by the addition of a calibrated bead suspension to the tube at the time of analysis.<sup>22-24</sup> The former method requires only one pipetting step, while the latter requires two. In both cases, accurate pipetting of sample and/or beads is required. The use of lyse-no-wash sample processing is mandatory in order to avoid loss of counting beads. This procedure allows the flow cytometer to measure the volume of sample analyzed, and then calculate the absolute number of any cellular element, relative to the known bead concentration. The use of these counting reagents for single-platform technique (SPT) has reduced variability between laboratories.<sup>11,20</sup> Consequently, SPT is preferred by an increasing number of laboratories.<sup>21</sup> The method detailed in Part B is a state-of-the-art application of SPT for T-cell subset analysis, and the method detailed in Part C describes its equivalent for CD34<sup>+</sup> cell enumeration.

## 7.5 Validation of SPT Procedures

### 7.5.1 Abnormal Lymphocyte Counts

Specimens with pronounced lymphopenia may not have sufficient lymphocytes in the whole blood aliquot for flow cytometric analysis. Conversely, normal concentrations of antibody reagents may be insufficient to saturate all binding sites in specimens with marked lymphocytosis, leading to inaccurate quantification (i.e., underestimation). Commonly available antibodies for lymphocyte subset enumeration will react appropriately when the WBC concentration is less than  $20 \times 10^9/\text{L}$ , assuming the reagents have been titrated to saturate their target antigens on 1 000 000 cells in 100  $\mu\text{L}$  of sample, as is common practice. Each laboratory must thus have a way of estimating the WBC concentration in the specimen, either by direct measurement or by having access to a simultaneous WBC concentration of the same patient from a hematology instrument. Samples with high WBC concentrations (i.e.,  $>20 \times 10^9/\text{L}$ ) should be diluted with phosphate-buffered saline (PBS) with 1% albumin or other protein, so that the WBC concentration is in the appropriate range for staining.

### 7.5.2 Precise and Accurate Pipetting Technique

Use of SPT to obtain absolute counts requires precise and accurate aliquoting of blood and counting beads. The reverse pipetting technique is recommended for dispensing these suspensions.

### 7.5.2.1 Verification of Pipetting Precision

The precision of pipetting should be evaluated periodically (e.g., monthly) to ensure the accuracy of results. All records of this evaluation procedure should be retained for quality assurance purposes.

- (1) Using the reverse pipetting technique, pipette ten replicates of blood and record the weights. Select a volume normally used in the performance of the assay.
- (2) Using the reverse pipetting technique, pipette ten replicates of bead suspension and record the weights (this applies to methods in which the beads must be pipetted into the tubes).
- (3) Calculate the mean, standard deviation, and coefficient of variation (CV). The CV for replicates should be < 2%.

### 7.5.2.2 Verification of Pipetting Accuracy

The following procedure can be used to verify how accurately the pipette measures volume. Here, a 100- $\mu\text{L}$  pipette is used as an example. Water is used for convenience because the weight of 1  $\mu\text{L}$  of water is 1  $\mu\text{g}$ .

- (1) Using the reverse pipetting technique, pipette ten replicates of distilled water and record the weight (100  $\mu\text{L}$  of water weighs 0.100 g).
- (2) Calculate the mean, standard deviation, and CV. The CV must be < 2% (range: 0.098 to 0.102 g).

### 7.5.2.3 Pipetting Procedure

The following information is derived from operational instruction manuals from several pipette manufacturers. For complete information and more detailed instructions, refer to the specific pipette instruction manuals of the manufacturers.

- (1) Select the desired volume (with manual pipettes, higher volumes should be set first; if adjusting from a lower to a higher volume, first surpass the desired volume and then slowly decrease the volume until the required setting is reached). Select an appropriate tip.
- (2) If applicable, select the desired mode (e.g., reverse pipetting). This mode is recommended for optimal precision and reproducibility.
- (3) Reverse pipetting can be done with a manual pipette by pressing the control button slightly past the first stop when aspirating, taking up more liquid than will be dispensed, then pressing the control button only to the first stop when dispensing. A small volume will remain in the tip after dispensing.

### ***Prerinsing***

The following procedures will help ensure optimal precision and accuracy.

- (1) Volumes  $\geq 10$   $\mu\text{L}$ : Prerinse pipette two to three times for each new tip (this involves aspirating and dispensing liquid several times). Reasons for prerinsing include the following:
  - to compensate for system pressure, for slight differences in temperature between pipette and liquid, and for properties of the liquid;
  - to clear the thin film formed by the liquid on the inside of the pipette. Without prerinsing, retention of a thin film on the inside wall of the tip would cause the first volume to be too small.

The thickness and nature of this film, and therefore the potential source of error, will vary depending on the nature of the liquid pipetted.

- (2) Volumes <10 µL: Do not prerinse the pipette, but rinse the tip after dispensing to ensure that the whole volume was dispensed. For smaller volumes, prerinsing is not recommended because the dispensed volume would be too great.

### ***Filling***

- (1) Make sure the tip is securely attached.
- (2) Hold the pipette upright.
- (3) When aspirating, keep the tip at a constant depth below the surface of the liquid.
- (4) Glide the control button slowly and smoothly (electronic pipettes perform this step automatically).
- (5) When pipetting viscous liquids (e.g., whole blood), leave the tip in the liquid for one to two seconds after aspirating before withdrawing it.
- (6) After liquid has been aspirated into the tip, do not lay the pipette on its side.

### ***Dispensing***

- (1) Hold the tip at an angle, against the inside wall of the vessel tube if possible.
- (2) Press the control button slowly and smoothly (electronic pipettes perform this step automatically).

#### **7.5.2.4 Other Recommendations for Precise and Accurate Pipetting**

- (1) To ensure optimal performance, the temperature of the fluid, the pipette, and the tips should be similar (volume errors may occur because of fluctuations in air displacement and viscosity of the liquid). Do not pipette liquids with temperatures >70 °C.
- (2) Volume errors may also occur with liquids that have a high vapor pressure or a density/viscosity that differs greatly from water. For the current purpose, water is most commonly used to calibrate pipettes and to check inaccuracy and imprecision (see Section 7.5.2.2).
- (3) Pipettes should be checked regularly for precision and accuracy.
- (4) Regular maintenance (including cleaning) should be performed either by the user or a service technician, according to manufacturer's instructions.

## **PART B: Enumeration of Lymphocyte Subsets**

### **8 Specimen Collection**

#### **8.1 Patient Information and Labeling of Specimens**

All samples submitted for testing must be labeled immediately at the time of draw with the full name of the patient, a unique patient identifier, test required, and date and time of draw. In addition, a test requisition should accompany all specimens. This should include a unique patient identifier, age, sex, date and time of specimen collection, name of requesting physician, and the source of the specimen (e.g., blood). A WBC concentration count should also be made available in case a dual-platform technique is used for lymphocyte subset enumeration (see Section 7).

#### **8.2 Venipuncture Technique**

Venipuncture procedures as outlined in the most current edition of CLSI/NCCLS document H3—*Procedures for the Collection of Diagnostic Blood Specimens by Venipuncture* should be followed when obtaining the blood specimen(s). Blood specimens must be regarded as potentially infectious. Blood collection procedures as outlined in the most current edition of CLSI document M29—*Protection of Laboratory Workers From Occupationally Acquired Infections* should be followed for the safety of the phlebotomist.

#### **8.3 Anticoagulant of Choice**

Ethylene-diaminetetraacetic acid (EDTA) or sodium heparin may be used. If a WBC concentration and differential are obtained from the same specimen used for FCM, EDTA is the anticoagulant of choice. Sodium heparin samples are stable for 48 to 72 hours and EDTA samples are stable for 12 to 24 hours. EDTA samples over 24 hours will become depleted of granulocytes.

### **9 Specimen Transport**

#### **9.1 Handling and Packaging of Specimens**

All specimens are handled in accordance with the understanding that they may transmit hepatitis virus, human immunodeficiency virus, or other infectious agents. The transportation of specimens containing potential infectious agents should include measures that minimize the risk of exposure of individuals to the potential etiologic agents. Specimens should be placed in well-stoppered test tubes, and the tubes should be placed with adequate absorbent material in a securely closed container for local transportation.

All specimens must be considered infectious and must be handled in such a way as to minimize risks for the laboratory worker and third-party carriers who transport specimens. Procedures for handling, packaging, labeling, and transporting specimens as defined by UN 602 must be followed.<sup>25</sup> Briefly, place specimen test tubes or vials (preferably unbreakable) in a leak-proof secondary container with sufficient absorbent material to absorb the entire contents of the vessel if breakage should occur. Specific labeling of specimens should be in accordance with state or federal (national) safety standards. In addition, local shipping regulations should be reviewed and followed when available.

Carefully wrap or package glass slides to eliminate sharp edges and corners. The secondary container should have sufficient insulating capacity to maintain transportation temperatures at room temperature (18 to 22 °C). Insulated containers of one-inch thickness are usually suitable for this purpose.

## 9.2 Effects of Storage and Holding

The yield of T-lymphocytes from blood samples is affected by several factors.<sup>26-31</sup> Ideally, specimens should be processed immediately after collection. When this is not possible, the laboratory should verify that the anticoagulant (see Section 8.3), holding temperature, and preparation method maintain specimen integrity similar to freshly processed material. This validation protocol should include normal and abnormal specimens.

Handling and transportation procedures must maintain the viability of the specimen as much as possible. Specimen integrity is the composite product of anticoagulant, storage conditions (time and temperature), and sample preparation procedure. Specimens can be maintained at room temperature (18 to 22 °C) and, ideally, they should be processed and fixed for flow cytometric analysis immediately after collection. Cold (4 °C) storage stabilizes CD34<sup>+</sup> cells and granulocytes, and may be useful for prolonged storage of some specimens. However, cold storage may selectively affect some lymphocyte subsets such as CD4<sup>+</sup> T-cells, and is therefore not preferred for lymphocyte subset analysis. Extreme temperatures must be avoided.<sup>32,33</sup>

## 10 Sample Preparation

The goal of sample preparation is to process a blood specimen into a representative sample suitable for introduction into the flow cytometer for analysis. All pertinent and representative cellular and antigenic parameters for lymphoid cells should be maintained. The RBC, which interfere with flow cytometric data acquisition due to their strong preponderance in numbers over WBC, should be removed. Avoidance of differential losses among lymphocyte subpopulations during cell preparation is critical. In general, the more a cell suspension is manipulated, the greater the opportunity for cell loss (which is seldom equivalent among all subpopulations of lymphocytes).

Therefore a whole blood, stain, lyse, no-wash method is recommended as the procedure of choice because it involves a minimum of manipulation.

### 10.1 Visual Inspection

Problems with specimen integrity are of two types:

- (1) Those indicating that the sample is altered or damaged and calling for immediate specimen rejection.
- (2) Those suggesting that specimen mishandling may have occurred and calling for further evaluation during sample preparation. Mishandling problems should be recorded because they may prove informative when preparing and analyzing the specimen and interpreting the results.

### 10.2 Hemolysis

Hemolysis indicates that the blood has been exposed to conditions that can cause RBC lysis, which implies that WBC may also be damaged. Consequently, hemolyzed specimens should be rejected.<sup>34</sup>

### 10.3 Clotted Blood

Even a partial clot may cause selective loss or alteration of certain subpopulations. All clotted samples do not reflect what was present *in vivo*. Clotted samples should be rejected unless irreplaceable, in which case every attempt should be made to obtain useful information. The clot can be removed and cells dispersed from the clot and filtered. A comment on the report should note that results may be affected.

## 10.4 Partial Draw

For some anticoagulants, a partially filled tube may produce hypertonic conditions deleterious to cells. Depending on the magnitude of the underfill, one may accept or reject the specimen. If the specimen is accepted, the laboratory should have previously demonstrated that the observed degree of underfill does not affect final results, and the underfill should be recorded for consideration during preparation, analysis, and interpretation.

## 10.5 Temperature Extremes

If the specimen tube has been transported to the laboratory from outside the institution, it may have been exposed to temperature extremes; however, examination upon receipt to determine if it is unusually warm or cold to the touch is not sufficient. For all deliveries, except those on the same day, it is recommended to include a minimum-maximum temperature sensor in the package to document whether or not extreme temperatures have been reached during shipment. Even if all other evaluation criteria are satisfactory, this observation should be noted for further consideration during preparation, analysis, and interpretation.

## 10.6 Improper Specimen Labeling

All samples submitted for testing must be labeled immediately at the time of draw with the patient's full name, a unique patient identifier, test required, and date and time of draw. If improper labeling is present, the laboratory should follow its established policy for dealing with unlabeled specimens and, if adequate identification cannot be obtained, the specimen should be rejected.

## 10.7 Specimen Integrity

One should visually inspect the specimen for any other abnormalities (e.g., lipemia). This observation should be noted for further consideration during sample processing, analysis, and interpretation.

## 10.8 Selection of a Sample Processing Procedure: Single-Platform Technique (SPT) Based on the Use of Counting Beads

This method is designed to enable determinations of both absolute and percentage lymphocyte subset counts using a single tube. The method is based on the addition of a number of fluorescent microspheres to a known volume of sample. This can be achieved in one of two ways: (1) using a tube containing a lyophilized pellet of a known number of fluorescent microspheres to which sample is added, or (2) by the addition of a calibrated bead suspension to the tube at the time of analysis. The former method requires only one pipetting step, while the latter requires two. In both cases, accurate pipetting of sample, reagent, and/or beads is required. This procedure allows the flow cytometer to measure the volume of sample analyzed, and from there the absolute number of any cellular element can be calculated, relative to the known bead concentration. The commercial availability of counting beads has resulted in decreased interlaboratory variability.<sup>19-21</sup> Consequently, SPT methods are preferred by an increasing number of laboratories.

### 10.8.1 Whole Blood Lysis

A whole blood, stain, lyse, no-wash method is required for SPT.

### 10.8.2 Selection of Lysing Agent

Several noncommercial lysing reagents are suitable. These include Tris-buffered ammonium chloride and hypotonic buffer.<sup>35,36</sup> Several proprietary lysing reagents are also available from instrument or

monoclonal antibody manufacturers. When using the latter reagents, the manufacturer's recommended protocol must be followed.

## 11 Immunostaining Panel

### 11.1 Reagents for Lymphocyte Immunophenotyping

A critical step in the immunophenotypic analysis of blood lymphocytes is the selection of the monoclonal antibodies. The selection of a specific clone and fluorochrome conjugate, as well as the antigen used for specific and sensitive identification of a given cell subset, represents a critical step for the success of this assay.

The following procedures assume the use of fluorochrome-conjugated antibody reagents.

| <u>Reagent</u>    | <u>Reactivity</u>                                  |
|-------------------|----------------------------------------------------|
| CD3               | T-lymphocytes                                      |
| CD4               | T-cell helper/inducer subset, monocytes            |
| CD8               | Cytotoxic T-cell subset, NK-lymphocyte subset      |
| CD19              | B-lymphocytes                                      |
| CD45              | All WBC                                            |
| CD16 <sup>a</sup> | NK lymphocytes, subset of monocyte/dendritic cells |
| CD56              | NK lymphocytes, subset of cytotoxic T-cells        |

The “Four-Color, Dual-Anchor” gating strategy identifies the major lymphocyte subsets (T, B, and NK), while providing several internal quality controls.<sup>2,37</sup> Together, these reagents should account for essentially all lymphocytes in a healthy individual. The laboratory's choice of reagents should be tailored to the clinical problem under investigation. It is recognized that other reagents or combinations may be useful for diagnosing or monitoring specific disease states. In some cases, more reagents than are clinically indicated or requested may require analysis to ensure that all relevant lymphocytes are included, and that a minimal number of irrelevant cell types are analyzed (see below). Single antibody determinations are not recommended.

Other reagents or combinations may be useful for diagnosing or monitoring specific disease states, which are beyond the scope of this document.

**Table 1. Recommended Antibody Panel for “Four-Color, Dual-Anchor” Gating Strategy**

| <b>Tube</b>    | <b>Antibodies</b>  | <b>Purpose of admixture</b>                                                                                                                |
|----------------|--------------------|--------------------------------------------------------------------------------------------------------------------------------------------|
| 1 <sup>a</sup> | CD3/CD19/CD56/CD45 | Gate on CD45 <sup>++</sup> and side scatter, count total T, B, and NK lymphocytes                                                          |
| 2 <sup>*</sup> | CD3/CD4/CD8/CD45   | Gate on CD45 <sup>++</sup> and side scatter, count CD3 <sup>+</sup> /CD4 <sup>+</sup> and CD3 <sup>+</sup> ,CD8 <sup>+</sup> T-lymphocytes |

<sup>\*</sup>The repeated use of CD3 serves as an internal control for tube-to-tube variability.

<sup>a</sup> Although CD16 and CD56 are often used simultaneously in a single color to increase the signal intensity of the CD16<sup>+</sup>, CD56<sup>±</sup> NK-cell subset, a CD56 mAb with a good signal-to-noise ratio makes adding of CD16 redundant. The exclusion of CD16 in the staining panel also avoids that an otherwise normal CD16<sup>+</sup> monocyte/dendritic cell subset, which may fall within the lymphocyte gate, would lead to inaccurate quantitation of NK cells.

## 11.2 Optimization of Staining Protocol

### 11.2.1 Volume

Use 50 to 400  $\mu\text{L}$  blood. Where sample plus reagent volume exceeds 0.2 mL, such as with lymphopenic samples (see above), mixing during incubation and/or an increased time of incubation may be required.

### 11.2.2 Temperature

Room temperature (18 to 22 °C) is recommended.

### 11.2.3 Time of Incubation With Reagent

Time to reach saturation is affected by sample volume and fluorochrome. Typical staining times recommended by the major manufacturers of monoclonal antibodies range between 10 and 30 minutes.

Any deviation from the standard laboratory or antibody manufacturer's protocol should be validated (i.e., it should have been documented that results similar to those using the standard procedure can be obtained).

### 11.2.4 Optimization of Reagent Concentration

Most commercially available monoclonal antibodies have recommended amounts for staining a defined volume of blood during a defined period of time. These recommended amounts are typically derived by determining the amount of reagent required to give:

- a minimal of (nonspecific) fluorescence on the negative population; and
- the maximum fluorescence intensity of the positive population.

For each batch of a given reagent, the laboratory should verify that a similar resolution between positive and negative signals and similar nonspecific and positive staining intensities are obtained, as with previous batches using the same method of sample preparation.

To validate any deviation from the manufacturer's recommended staining conditions (e.g., time, volume, temperature, or cell number), the laboratory must determine the minimal amount of reagent required to give optimal resolution between positive and negative signals, and optimal positive staining intensity.

**NOTE:** Excess reagent may cause increased nonspecific staining of negative cell populations, resulting in loss of resolution, while staining using too large amounts of monoclonal antibody may cause quenching of fluorescence signals, resulting in reduced staining intensities and proportions of positive cells.

## 12 Sample Quality Control Procedures

### 12.1 Control Specimen(s)

An isotype control staining is not needed.<sup>1,38,39</sup>

#### 12.1.1 Positive Procedure Control

A positive control sample serves to determine whether procedures for preparing and processing the specimens are adequate. Prepare this control on each occasion that patient specimens are prepared. A

long-term stabilized blood specimen validated for this purpose is used. If the positive procedure control falls outside established predefined target ranges, the reason for this deviation must be determined.

### 12.1.2 Positive Control for Evaluating Reagents

Use a whole blood specimen from an apparently healthy donor as a positive control to test the labeling efficiency of new batches of monoclonal antibodies, or when the labeling efficiency of the current batch is questioned. Prepare this control only as needed (i.e., when antibodies must be evaluated) in parallel with a batch of the same antibody with documented acceptable performance.

**NOTE:** Alternative monoclonal antibodies (i.e., alternative clones, conjugates, and/or manufacturers) must demonstrate similar results to those of known acceptable performance. Use fresh whole blood for this purpose, or an alternative source of lymphocytes (e.g., commercial long-term stabilized blood or cryopreserved mononuclear cells).

## 13 Sample Analysis

### 13.1 Instrument Configuration

Quality control of instrument setup and performance under the test conditions for enumerations of lymphocyte subset enumeration are detailed in Appendix A. The instrument should operate under conditions that minimize biohazard to the operator and maximize decontamination of fluid waste.

### 13.2 Verification of Acceptable Specimen Viability

Nonviable cells are a significant source of false positive staining and each laboratory should have an established method for assessing sample viability after preparation and staining.<sup>40-42</sup> Viability testing is only recommended for samples tested more than 24 hours after drawing, or if visual inspection suggests that samples have been deteriorated otherwise.

To assess viability, a separate staining using 7-aminoactinomycin D (7-AAD) counterstained with CD45 (to assess cell death among lymphocytes, monocytes, and granulocytes) is recommended (see Part C).

A minimum viability of 75% is recommended.<sup>35,43</sup> For irreplaceable samples with a lower viability, the staining panel shown in Table 1 may be adapted as follows:

- (1) 7-AAD + CD45 + CD3 + CD4;
- (2) 7-AAD + CD45 + CD3 + CD8;
- (3) 7-AAD + CD45 + CD3 + CD16 & 56;
- (4) 7-AAD + CD45 + CD3 + CD19.

Test results obtained in this way must be flagged as such.

### 13.3 Verification of Representative Sampling

To avoid possible artifacts resulting from differential sedimentation rates, samples should be well-mixed immediately before introduction into the instrument. Verification of representative sampling is relevant when the light scatter characteristics of the stained samples are abnormal, since this observation may indicate size and/or density differences between stained cells that could affect relative sedimentation rates. Representative sampling may be verified by analyzing a well-mixed sample and comparing it to results of analysis of the same sample that has been allowed to settle for a time comparable to the longest expected sample analysis time.

### 13.4 Establishment and Monitoring of “Lymphocyte” Gate During Flow Cytometric Data Acquisition

The first step in flow cytometric data acquisition is to establish the gating parameters that will be used in distinguishing lymphocytes from other cell types in the specimens. The lymphocyte population is identified as having bright CD45 fluorescence and low side-scatter properties (Figure 1, panel A). A threshold or discriminator is set on CD45 to exclude CD45<sup>-</sup> events (i.e., cellular “debris”). The photomultiplier tube (PMT) setting for sideward light scatter may be adjusted so that all WBC are visible. A “gate” or “region” is then drawn on the bright CD45<sup>+</sup> cell population, in order to determine when data acquisition can be stopped.

Ungated list-mode data containing at least 5000 lymphocytes are acquired from each staining to ensure that enough cells have been counted, to provide an accurate analysis of even small lymphocyte subsets (i.e., 10% of total lymphocytes).

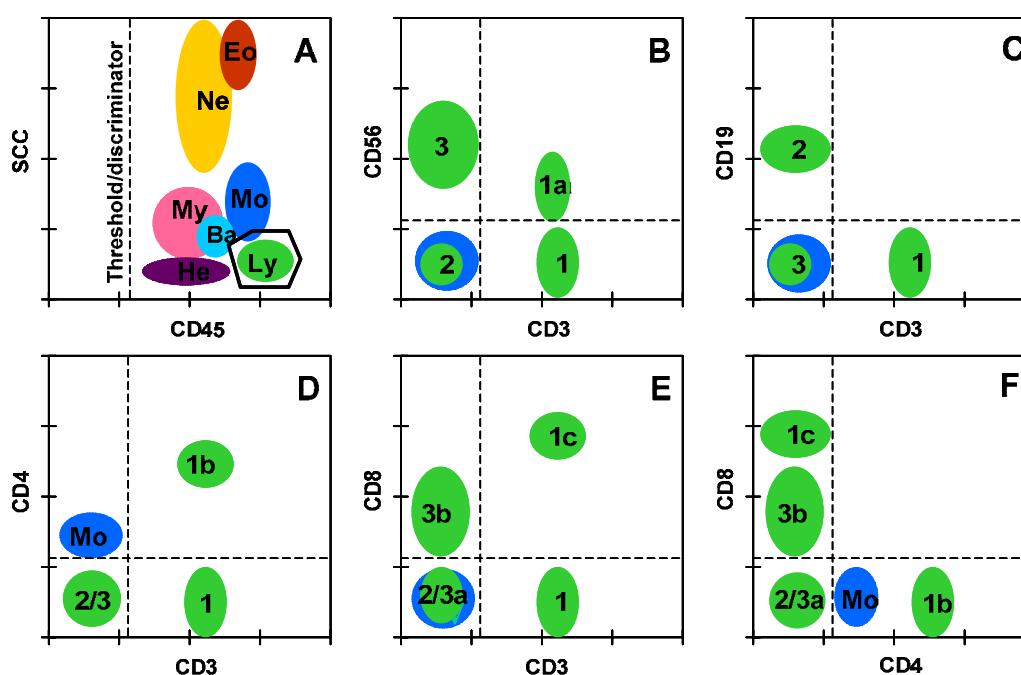


**Figure 1. Typical Illustration of a Four-Color, Two-Tube Lymphocyte Subset Enumeration Assay Using Bivariate Histograms (Dot Plots).** Tube 1 (panels A, B, and C) has been stained with a combination of CD3, CD19, CD45, and CD56 mAb. Tube 2 (panels D, E, and F) has been stained with a combination of CD3, CD4, CD8, and CD45 mAb. Panel A shows the CD45 vs. side scatter plot of ungated WBC with a threshold/discriminator set on CD45<sup>-</sup> events to exclude debris. Lymphocytes (Ly) are depicted in green, monocytes (MO) in blue, eosinophils (Eo) in red, neutrophils (Ne) in orange, basophils (Ba) in Cyan, hematogones or pre-B-cells (He) in purple, and myeloid blasts (My) in pink. Panels B to F show the analysis of the contents of the gate or region drawn around the lymphocytes in panel A, which may also include a small proportion of monocytes. The following lymphocyte subsets can be identified: CD3<sup>+</sup> T-cells (indicated with “1”), CD3<sup>+</sup>,56<sup>+</sup> T-cell subset (“1a”), CD3<sup>+</sup>,4<sup>+</sup> T-cell subset (“1b”), CD3<sup>+</sup>,8<sup>+</sup> T-cell subset (“1c”), CD19<sup>+</sup> B-cells (“2”), CD3<sup>-</sup>,CD56<sup>+</sup>,8<sup>-</sup> NK cells (“3a”) and CD3<sup>-</sup>,CD56<sup>+</sup>,8<sup>+</sup> NK cells (“3b”).

## 14 Data Analysis

### 14.1 CD45 Gating

In the first step, the lymphocytes are selected, which are identified as having bright CD45 expression and low sideward light scatter properties (Figure 1, panel A). In this analysis, care must be taken to include **all** lymphocytes (first priority), while monocytes (slightly lower CD45 expression than lymphocytes, and intermediate SSC) and basophils (slightly lower CD45 expression than lymphocytes, and low SSC) should be excluded as much as reasonably possible (second priority). Within the lymphocytes, CD45 expression may be slightly less on B-lymphocytes than on T-lymphocytes, the latter typically the major lymphocyte subset. NK lymphocytes have similar intensity of CD45 expression as T-lymphocytes, but have a slightly higher SSC signal.

Usually, CD45/SSC gates on lymphocytes set in this way will contain > 95% of the lymphocytes. In addition, lymphocyte purity is typically high with this gating strategy; therefore, correction of lymphocyte subset values for contamination with other WBC will not be needed.<sup>39</sup> By virtue of the use of the CD3 T-cell lineage marker, inclusion of a (small) proportion of monocytes will not affect the subtyping of the CD3<sup>+</sup> T-cells; the impact of contamination of the lymphocyte gate by monocytes will only affect the proportion of the T-cell subsets when these are expressed as percentage of cells within the lymphocyte gate (see Figure 1, panels B to F).

If an estimate of lymphocyte subset recovery is needed (i.e., expressed as percentage of total lymphocytes within the CD45/side scatter gate), T, B, and NK cells must all be enumerated.

**NOTE:** Validation of the CD45, SSC gate is recommended during its initial use to help determine the CD45 and SSC characteristics of T, B, and NK cells and to ensure their inclusion in the lymphocyte gate. To visualize the CD45 and SSC characteristics of T, B, and NK lymphocytes, these subsets can be plotted in a bivariate CD45, SSC histogram after selection on the basis of their CD3 and SSC, CD19 and SSC, and CD56 and SSC characteristics, respectively.

### 14.2 Lymphocyte Subset Analysis

Fluorescence cursors are set based on the histograms displaying CD3 vs. CD56, CD3 vs. CD19, CD3 vs. CD4, and CD3 vs. CD8, respectively, so that the negative and positive populations in these histograms are unequivocally distinguished (see Figure 1, panels B to E).

Each patient or control specimen is analyzed using the lymphocyte gate and subset cursor settings for that particular patient or control.

The following controls for analytic reliability may be used:

- With SPT, the accuracy of the absolute count may be verified as follows. The parameter “acquisition time” may be used to determine how long it takes to acquire the number of counting beads that represents a defined volume of blood analyzed. For this purpose, “time” must be set as an active parameter during data acquisition. If blood pipetting is performed without noticeable error and the counting beads are accurately added to the tubes (where applicable), the time required to analyze a *predefined number* of counting beads should be constant. If the time required to accumulate this number varies significantly between specimens, a serious problem with sample preparation must be suspected, and specimen processing should be repeated.
- In most cases, the sum of the percentages of CD3<sup>+</sup>,CD4<sup>+</sup> and CD3<sup>+</sup>,CD8<sup>+</sup> cells should equal the total percentage of CD3<sup>+</sup> cells  $\pm 5\%$ , with a maximum variability of  $\pm 10\%$ . However, for specimens

containing a considerable number of unusual T-cell subsets (e.g., TCR- $\gamma\delta^+$  T cells, CD3<sup>+</sup>,CD4<sup>-</sup>,CD8<sup>-</sup> or CD3<sup>+</sup>,CD4<sup>+</sup>,CD8<sup>dim</sup> T cells), this reliability check may exceed the above limits.

### 14.3 Analysis of Counting Beads (SP methods only)

To enumerate the absolute numbers of a certain cell population (which is the concentration per unit of volume) by a single flow cytometric measurement (i.e., SP method), an accurately measured volume of sample is pipetted into a tube, and the same accurately measured volume of counting beads is added. SP assays may also be performed using commercially available absolute counting tubes containing a lyophilized pellet with a known number of counting beads.

During data analysis, either singlet beads must be included in the analysis, or all beads (singlets and aggregates) are included in the bead count as per the manufacturer's instructions.

## 15 Data Storage

The possibility of patients or their legal representatives contesting diagnostic implications derived in part from lymphocyte phenotypic analysis requires that the laboratory is able to demonstrate and verify the process used in arriving at the reported test results. Both the data stored and the methods used to obtain the data must be thoroughly documented.

### 15.1 Information Storage

Store list-mode data for all specimens analyzed. This policy allows for a comprehensive reanalysis of the raw data. At a minimum, retain hard copies of the CD45, SSC lymphocyte gate, and the bivariate histograms showing the lymphocyte subset analysis results of each sample.

Retain all primary files, worksheets, and report forms for two years or as required by state or local regulation, whichever is longer. Data can be stored electronically (see above); backup of the data shall be in place to prevent loss of patient data in case of hardware or software failure.<sup>44</sup> Disposal after the retention period is at the discretion of the laboratory director.

QA/QC data files should include all parameters, analysis regions, and analytical results used to verify system (instrument and method) performance.

### 15.2 Types of Data Storage

#### 15.2.1 Paper Hard Copy

If data are stored as paper hard copy, all histograms and analysis regions used in arriving at the reported test results (e.g., lymphocyte gates, analysis boundaries) must be recorded on the hard copy of the relevant data plot. A unique specimen identifier should also be recorded with each data plot.

#### 15.2.2 Archival Files

A variety of data storage media may be used, depending on the particular systems available in the laboratory, including floppy disks, removable hard disks, optical disks, and magnetic tape.

#### 15.2.3 Duration of Data Storage

Patient data should be kept for a minimum of two years, or as required by state and federal (national) law, whichever is longer. After that, it is the responsibility of the laboratory director to determine whether to

save or discard the data. QA/QC data should be kept for a minimum of two years.

Data may be stored for as long thereafter as the laboratory director considers necessary, providing that storage space is available.

## 16 Data Reporting and Interpretation

### 16.1 Worksheet

A standard laboratory worksheet should be developed for reporting immunophenotyping data.

Report all data in terms of CD designation with a short description of what that designation means (see Section 11.1).

### 16.2 Supervisory Check

Recording of laboratory data and calculations should be checked by supervisory personnel, and authorized.

### 16.3 Review of Data Displays

Data displays showing the discriminator/threshold setting during data acquisition, the number of events acquired, the light scatter patterns and antibody staining profiles, and all analytical gates and fluorescence cursor settings used to arrive at the test result should be reviewed by a laboratory professional when interpreting the data.

### 16.4 Reporting of Data

Absolute numbers and percentages are reported for each of the lymphocyte populations (i.e., T, B, and NK cells), and for the T-cell subpopulations (i.e., CD3<sup>+</sup>, 4<sup>+</sup> and CD3<sup>+</sup>,8<sup>+</sup>). Percentages are particularly important in the monitoring of pediatric patients.

These calculations may be generated directly by software that reports calculated results using either a single- or dual-platform technique.

When using a DP technique, an accurate WBC concentration should be obtained. This count should be performed on a sample drawn at the same occasion as the specimen drawn for phenotyping, and determined within six hours (see the most current edition of CLSI/NCCLS document H20—*Reference Leukocyte [WBC] Differential Count [Proportional] and Evaluation of Instrumental Methods*). When an accurate WBC concentration is not available, absolute lymphocyte counts cannot be reported using a dual-platform technique, and a single-platform technique must be used.

**NOTE:** For DP methods, the WBC and not the absolute lymphocyte count should be used to calculate absolute T-cell subset counts. This requirement is caused by the fact that WBC is a much more stable denominator than the absolute lymphocyte count, which shows a larger variation between hematology analyzers.

With SP methods, the absolute counts are directly derived from the flow cytometric data. These calculations may be generated directly by dedicated software. For manual data processing, the following formula should be used to calculate the absolute lymphocyte count:

$$\frac{\text{No. of events in the bright CD45 region}}{\text{No. of events in the microfluorosphere region}} \times \frac{\text{Total no. of microfluorospheres added}}{\text{Volume of blood added}}$$

## 16.5 Interpretation of Data and Inclusion of Reference Intervals

Data interpretation should be made using an established laboratory reference interval, which should be included on the final report (see Section 17).

## 16.6 Flagging of Test Results With Out-of-Range Control Samples

If the results of analysis of positive method control samples are outside the laboratory reference interval and no reason has been determined, results on patient specimens prepared at the same time are potentially suspect and this should be noted on the test report.

## 17 Reference Intervals

Reference intervals should be obtained in a manner similar to that for the patient material. Reference intervals for immunophenotyping test results must be determined for each laboratory. Separate reference intervals must be established for adults and children, and the appropriate ranges must be reported for patient specimens. See the most current edition of CLSI/NCCLS document C28—*How to Define and Determine Reference Intervals in the Clinical Laboratory*.

## **PART C: Enumeration of CD34<sup>+</sup> Hematopoietic Stem and Progenitor Cells**

### **18 Specimen Collection**

#### **18.1 Patient Information and Labeling of Specimens**

All samples submitted for testing must be labeled immediately at the time of draw with the full name of the patient, a unique patient identifier, source of specimen, and date and time of draw. In addition, a test requisition should accompany all specimens. This should include a unique patient identifier, age, sex, date and time of specimen collection, name of requesting physician, source of the specimen (e.g., blood), test required, and relevant clinical information. Information such as WBC concentration and differential should also be made available, in case a dual-platform technique is used for CD34<sup>+</sup> cell enumeration (see Section 7.2).

#### **18.2 Venipuncture Technique**

Venipuncture procedures as outlined in the most current edition of CLSI/NCCLS document H3—*Procedures for the Collection of Diagnostic Blood Specimens by Venipuncture* should be followed when obtaining the blood specimen(s). Blood specimens must be regarded as potentially infectious. Blood collection procedures as outlined in the most current edition of CLSI document M29—*Protection of Laboratory Workers From Occupationally Acquired Infections* should be followed for the safety of the phlebotomist.

#### **18.3 Anticoagulant of Choice**

Ethylene-diaminetetraacetic acid (EDTA) is used for blood specimens. Apheresis specimens are typically anticoagulated with acid citrate dextrose (ACD solution A). EDTA, heparin, or ACDA may be used for cord blood and bone marrow specimens, although specimen stability varies somewhat between these anticoagulants.<sup>26,45-48</sup> If a WBC concentration and differential are obtained from the same specimen used for FCM (i.e., dual-platform method), EDTA is the anticoagulant of choice. In any other circumstance (such as apheresis and cord blood specimens), absolute CD34<sup>+</sup> cell counts should be generated using single-platform methodology.

### **19 Specimen Transport**

#### **19.1 Handling and Packaging of Specimens**

All specimens are handled in accordance with the understanding that they may transmit hepatitis virus, human immunodeficiency virus, or other infectious agents. The transportation of specimens containing potential infectious agents should include measures that minimize the risk of exposure of individuals to the potential etiologic agents. See Section 9.1 for more details.

#### **19.2 Effects of Storage and Holding**

The yield of CD34<sup>+</sup> cells from blood, cord, bone marrow, and apheresis samples is affected by a number of factors.<sup>49</sup> Ideally, specimens should be processed immediately after collection. When this is not possible, the laboratory should verify that their anticoagulant (see Section 18.3), storage temperature, and preparation methods maintain specimen integrity comparable to freshly processed material. This validation protocol should include normal and abnormal specimens.

Unlike some T-cell subsets, CD34<sup>+</sup> cells and granulocytes are maximally preserved when refrigerated. Specimens should be maintained at 2 to 6 °C during transportation and storage for maximum flexibility in sample preparation methods.<sup>50</sup>

## 20 Sample Preparation

The goal of sample preparation is to process blood, cord blood, bone marrow, or apheresis specimens into a representative sample suitable for introduction into the flow cytometer for analysis. All pertinent and representative cellular and antigenic parameters for CD34<sup>+</sup> cells should be maintained. Irrelevant cells (e.g., RBC) that may slow or interfere with analysis should be for the most part removed. Avoidance of differential losses among leukocyte subpopulations during cell preparation is critical, as such losses may bias the outcome of CD34<sup>+</sup> enumeration assays. In general, the more a cell suspension is manipulated or processed, the greater the opportunity for cell loss. Such losses are seldom equivalent among all subpopulations of WBC. A whole blood stain, lyse, no-wash method is recommended as the procedure of choice because this procedure involves a minimum of sample manipulation.

### 20.1 Visual Specimen Evaluation

Observable specimen problems are of two types:

- (1) Those indicating that the sample is altered or damaged and calling for immediate specimen rejection.
- (2) Those suggesting that specimen mishandling may have occurred and calling for further evaluation during sample preparation. Mishandling problems should be recorded because they may prove informative when preparing and analyzing the specimen and interpreting the results.

### 20.2 Specimen Integrity

Hemolysis indicates that the blood has been exposed to conditions that can cause RBC lysis, suggesting that the hematopoietic stem cells may also be damaged. Severely hemolyzed blood specimens should be rejected. Exceptions to this are samples of cord blood or RBC containing apheresis products that have been previously frozen. Cryopreservation does not protect the RBC population and the RBC will be hemolyzed on thawing. This does not impact the assay of CD34<sup>+</sup> cells in these products. One should visually inspect the specimen for any other abnormalities indicating compromised integrity. Such observation should be recorded for further consideration during processing, analysis, and interpretation.

### 20.3 Clotted Blood

Even partial specimen clotting may cause selective loss or alteration of certain subpopulations. Clotted specimens should be rejected whenever possible. Occasionally, small clots may be present in samples from apheresis packs. These samples represent the actual product that will be given to the patient and are therefore considered as irreplaceable (see Section 5.1). In such cases, clots should be removed by filtering before testing, and a note of the fact that the sample was partially clotted should be included in the report.

### 20.4 Partial Draw

When testing blood, for some anticoagulants, a partially filled tube may produce hypertonic conditions deleterious to cells (i.e., ACD). Depending on the magnitude of the underfill, the laboratorian may accept or reject the specimen. If the specimen is accepted, the laboratory should have previously demonstrated that the observed degree of underfill does not affect final results, and the underfill should be recorded for consideration during preparation, analysis, and interpretation.

## 20.5 Temperature Extremes

If the specimen tube has been mailed to the laboratory, it may have been exposed to temperature extremes and should be examined upon receipt to determine if it is unusually warm or cold to the touch. Even if all other evaluation criteria are satisfactory, this observation should be noted for further consideration during preparation, analysis, and interpretation.

## 20.6 Improper Specimen Labeling

Any specimen that has not been labeled with a unique patient identifier or specimens labelled with contradictory patient information (i.e., mismatch of patient name and unique identifier) should be rejected. As apheresis products are considered irreplaceable, any decision to reject samples from these products must be done in consultation with the appropriate medical staff.

## 20.7 Specimen Age and Storage Conditions

Maximum acceptable specimen age is a function of the anticoagulant, lysing agent, and storage conditions including cell concentration.<sup>51-53</sup> The laboratory should verify the maximum allowable specimen age for the anticoagulant and lysing reagent combination used. While specimens should be tested immediately as a rule, it is recognized that this may not always be possible. Specimens that cannot be tested immediately should be maintained at 2 to 6 °C to preserve viability. If specimens are stored, holding at 2 to 6 °C is recommended. The laboratory must verify that stored specimens give results equivalent to fresh specimens using their storage conditions and lysing procedure.

Following sample preparation and immunostaining, samples should be stored on ice and analyzed as soon as is practical, and definitely within one hour. Cord blood, bone marrow, and thawed samples are more susceptible to increased cell death after preparation, due to the already high content of dead cells and debris. Consequently, FCM of these samples should be performed immediately.

## 20.8 Selection of a Sample Preparation Procedure

### 20.8.1 Whole Blood Lysis vs. Density Gradient Methods

A whole blood lysis method is recommended because it does not employ density gradients or lengthy centrifugation steps, that may lead to differential losses of specific subpopulations. Whole blood lysis assumes equivalent recovery of all leukocyte subsets. Cryopreserved specimens do not require RBC lysis.

### 20.8.2 Selection of Lysing Agent

Several lysing techniques are available. These include Tris-buffered ammonium chloride and hypotonic buffer.<sup>35,36</sup> Several proprietary lysing reagents are also available from instrument or monoclonal antibody manufacturers. When using commercial reagents, the manufacturer's recommended protocol must always be followed.

**NOTE:** When using nonfixing lysing reagents, such as ammonium chloride, it is essential that stained samples are kept on ice until flow cytometric analysis. This analysis should take place within one hour of sample preparation. Viability assessment is performed on unfixed samples. Laboratory-induced cell death can be caused by improper storage and handling of samples after preparation.

## 21 Immunofluorescence Staining of CD34 and CD45 Surface Antigens

### 21.1 Reagents

#### 21.1.1 Monoclonal Antibodies for CD34<sup>+</sup> Hematopoietic Stem Cell Enumeration

The following procedures assume the use of fluorochrome-conjugated antibody reagents. Due to the Boolean, sequential gating strategy and fluorescence intensities of the recommended markers, directly labeled subclass controls are redundant, as the analytical strategy is capable of correcting for increased levels of autofluorescence and nonspecific mAb binding.

| <u>Reagent</u>                | <u>Reactivity</u>                       |
|-------------------------------|-----------------------------------------|
| CD34                          | Hematopoietic stem and progenitor cells |
| CD45                          | Pan-leukocyte marker                    |
| 7-amino-actinomycin D (7-AAD) | Viability dye                           |

CD34 reagents that bind to class III epitopes should be used because they detect all glycoforms of the CD34 molecule, which is expressed on hematopoietic stem and progenitor cells. The CD45 reagent must be capable of detecting all isoforms and glycoforms of CD45, which are expressed on all WBC, with the exception of some plasma cells and some acute lymphoblastic leukemia cells.<sup>9</sup> 7-AAD is an impermeant viability dye that has a low quantum yield when unbound, but which increases dramatically when bound to the DNA of cells that no longer have an intact membrane. As such, it is an excellent viability dye for this application, due to the low background fluorescence of 7-AAD-negative (i.e., viable) cells.

Particularly for the analysis of heavily processed and post-thawed samples, some studies have included other dyes to exclude apoptotic cells in addition to nonviable 7-AAD<sup>+</sup> cells. However, it is unclear whether the appearance of these apoptotic events are due, at least in part, to the extra processing involved (i.e., ‘laboratory-induced apoptosis’) or truly reflects the status of the sample before processing.<sup>54</sup> Therefore, the 7-AAD method is recommended as a satisfactory means to exclude dead cells for all routine testing. The application as described in this document is a three-color analysis with fluorescent beads to allow absolute viable counts in a single tube (see Appendix A, Section A2). Although other reagents or combinations may also prove useful for CD34<sup>+</sup> cell analysis, single antibody determinations are not recommended under any circumstances.

Addition of other reagents or combinations to identify subsets of CD34<sup>+</sup> cells may be useful for diagnosing or monitoring of certain disease states. However, the additional benefit of CD34 subset enumeration to predict engraftment potential has not been demonstrated and this topic is, therefore, beyond the scope of this document.

### 21.2 Optimization of Staining Protocol

When commercially available kits of CD45 and CD34 mAb have a detailed staining protocol, the protocol should be followed exactly. This is also true for RBC lysis reagents.

#### 21.2.1 Volume

Antibody and reagent volumes should not exceed a total of 200  $\mu$ L to allow for optimal staining conditions.

### 21.2.2 Temperature

Room temperature (18 to 22 °C) is recommended for staining samples for immunophenotyping. After staining, samples should be stored at 4 °C until analysis in order to preserve cell viability.

### 21.2.3 Time of Incubation With Reagent

Time to reach saturation is affected by various factors including sample volume and fluorochrome, antibody affinity, and nature of the epitope). PE-conjugated reagents may require longer incubation times compared with FITC conjugates. Typical staining times recommended by the manufacturer vary from 10 to 30 minutes.

Any deviation from the standard laboratory or antibody manufacturer's protocol should be validated (i.e., it should have been documented that results similar to those using the standard procedure can be obtained).

### 21.2.4 Cell Count

The amount of antibody recommended for staining with most commercial reagents will be sufficient to stain 100 µL of blood, assuming a normal leukocyte count of approximately 4 to 10 x 10<sup>9</sup> WBC/L. However, there is usually sufficient leeway to provide adequate staining of blood samples with higher WBC counts. The upper limit of the WBC count that can be accepted is determined by the leukocyte count in the sample, and should not be higher than 20 x 10<sup>9</sup>/L unless it is clearly stated by the manufacturer of the CD45 antibody that higher levels can be safely analyzed. As CD34<sup>+</sup> cells represent a small population of total WBC (usually < 1%), it is unlikely that saturation issues will be significant for the CD34 antibody, even with high leukocyte counts. For samples with high leukocyte counts (e.g., mobilized blood or apheresis products), dilution to the appropriate staining range should be performed in phosphate buffered saline with 1 to 2% protein added.

## 22 Sample Quality Control Procedures

### 22.1 Negative Reagent Control

Negative reagent controls are of limited, if any, utility in the enumeration of rare events, such as CD34<sup>+</sup> cells. Some isotype controls can stain more cells nonspecifically, than are stained specifically by a CD34 conjugate. Indeed, studies of a large number of normal hematopoietic samples have shown that the sequential gating approach best delineates specific from nonspecific staining, and that traditional isotype controls provide no useful information regarding the levels of nonspecific staining in the flow cytometric analysis of rare events.<sup>55,56</sup> For this reason it is recommended that isotype controls are not used. In this context, it is relevant that analysis of a normal blood sample that has a range of CD34<sup>+</sup> cells from <1 to 7 cells/µL provides far greater information on cell and reagent parameters than an isotype control.

### 22.2 Positive Reagent Control

Fresh blood samples, commercial stabilized whole blood samples, or CD34<sup>+</sup> cell lines may be used as reagent controls. Positive reagent controls are used to validate proper binding of current reagent lots and to test new reagent lots, and should be run when reagent lots are changed. The accepted variability for new lots of reagents compared to current lots should not be any greater than the variability found for replicate samples of the current lot.

One advantage of commercial controls or cell lines is that they provide a cell source that is consistent from day to day for comparison of reagent performance. An advantage of this type of preparation is that it can also double as a positive procedure control when the whole blood procedure is the method used for

patient specimens. A disadvantage of such controls is that the ‘preserved’ controls are by definition nonviable, and this is taken into account when viability dyes are in regular use in the assay. Further, preserved controls exhibit different light scatter characteristics to those of fresh samples, and routine instrument settings may be suboptimal for their analysis.

If commercially available controls are used, carefully follow the manufacturer’s directions.

### **22.3 Positive Procedure Control**

A positive control sample serves to determine whether procedures for preparing and processing the specimens are adequate. Prepare this control on each occasion that patient specimens are prepared. Use a long-term stabilized blood specimen validated for this purpose. If the positive procedure control falls outside established predefined target ranges, the reason for this deviation must be determined.

## **23 Sample Analysis**

### **23.1 Instrument Configuration**

Quality control of instrument setup and performance under the test conditions for enumerations of lymphocyte subset enumeration are detailed in Appendix A. The instrument should operate under conditions that minimize biohazard to the operator and maximize decontamination of fluid waste.

### **23.2 Order of Analysis**

#### **23.2.1 Procedural Control Specimen**

- (1) Color compensation controls.
- (2) Controls for establishing and monitoring light-scatter gates used to define WBC.
- (3) Positive controls for verification of staining and preparation methods.

#### **23.2.2 Patient Specimens**

- (1) Controls for establishing and monitoring light scatter gates used to define WBC.
- (2) Patient samples stained with monoclonal antibodies.

### **23.3 Verification of Acceptable Specimen Viability**

Because of potential biohazards, it is generally recommended that all samples be fixed using agents known to inactivate viruses and other infectious agents before introduction into the flow cytometer (see Section 6 on safety). However, nonviable cells are a significant source of false-positive staining, and each laboratory should have an established method for assessing sample viability after preparation and staining.<sup>39-42</sup> When fluorescent viability stains such as 7-aminoactinomycin D (7-AAD) are used to monitor sample viability on the basis of dye exclusion, it is essential to use unfixed samples, since these dyes may redistribute over all cells after fixation and storage. If alternative viability probes are used for postfixation viability discrimination, the laboratory must verify, using representative patient specimens, that such probes give results similar to those obtained using standard viability dyes (such as propidium iodide or trypan blue) in unfixed samples. Whatever method is used, the laboratory is responsible for determining the concentration of viability dye to use to give optimal resolution between live and dead cells.

Laboratories should establish their own lower limit of viability for acceptance of each sample type. For samples that have not been cryopreserved, a minimum viability of 75% is recommended.<sup>35,43</sup> It is recognized that due to methods of collection, this may not be possible for cord blood or bone marrow samples or samples that have been thawed. If specimen viability is below the established laboratory minimum, test results are suspect and this should be noted on the test report. If low viability is suspected, due to delay in sample analysis after processing, a fresh sample should be prepared.

### 23.4 Verification of Representative Sampling

To avoid possible artifacts resulting from differential sedimentation rates, samples should be well-mixed immediately before introduction into the instrument. Verification of representative sampling is relevant when the light scatter characteristics of the stained samples are abnormal, since this observation may indicate size and/or density differences between stained cells that could affect relative sedimentation rates. Representative sampling may be verified by analyzing a well-mixed sample and comparing it to results of analysis of the same sample that has been allowed to settle for a time comparable to the longest expected sample analysis time.

## 24 Enumeration of Absolute Numbers of CD34<sup>+</sup> Cells

### 24.1 Introduction

Counterstaining CD34 with CD45 mAb allows the elimination of debris and nonspecifically stained events from the analysis, as well as the generation of a reliable denominator (i.e., WBC) against which to measure CD34<sup>+</sup> cells. Importantly, this approach also allows the discrimination of hematopoietic progenitor cells (that express relatively low levels of CD45 on their surface) from lymphocytes and monocytes (that express high levels). Just as lymphocytes, monocytes, and granulocytes form discrete clusters on bivariate plots of CD45 vs. sideward light scatter (SSC), so do nonmalignant CD34 hematopoietic stem and precursor cells. This method, which originally has been published as the “ISHAGE protocol” (International Society for Hematotherapy and Graft Engineering),<sup>9</sup> uses the maximum information available from four parameters—forward light scatter (FSC), SSC, CD34 expression, and *intensity* of CD45 expression. These four parameters are combined in a sequential Boolean gating strategy that can be used on blood, apheresis products, purified CD34<sup>+</sup> cell suspensions, cord blood, and bone marrow specimens. With the modifications outlined in Sections 7.4 and 14.3, the ISHAGE Protocol<sup>9</sup> is converted into a single-platform assay.<sup>5</sup>

Exclusion of dead cells from CD34<sup>+</sup> cell enumeration can be achieved by inclusion of the DNA G-C intercalating dye 7-AAD, which allows discrimination between viable and nonviable (CD34<sup>+</sup>) cells. This modification is simple to apply and may be of value in all sample types. It is highly recommended for apheresis samples over four hours old, all “fresh” cord blood and bone marrow samples, or samples that have been manipulated in any manner, and is essential for the accurate analysis of samples that have been previously frozen. 7-AAD is excited at 488 nm and has maximum emission at 660 nm. Consequently, the dye can be used in single-laser systems with standard FITC and PE fluorochromes. Compensation for spectral overlap between PE and 7-AAD is addressed in Appendix A.

This procedure requires reverse pipetting of the sample and counting beads. In order to make this guideline optimally useful in the laboratory, the writing committee has chosen to provide specific protocol descriptions for two major groups of instruments and software, referred to as *Group A* and *Group B*. Evidently, this protocol can be adapted for other equivalent brands and types of flow cytometers and software with similar specifications (i.e., featuring blue laser emission at 488 nm and three fluorescence parameters).

## 24.2 Instrument Setup

Position the FSC, SSC, and fluorescence (FL) windows of analysis and adjust electronic correction for spectral overlap according to standard procedures for lyse-no-wash immunophenotyping assays.

**NOTE:** Depending on the manufacturer, counting beads are detected on Group B instruments at lower FSC levels than lymphocytes; in such cases, the FSC threshold must be set below that of singlet beads so that minor differences in sample preparation methods do not result in the exclusion of any beads from acquisition (see Figure 3, histogram 7).

### 24.2.1 Creation of Bivariate Histograms (Dot Plots)

Histogram 1: CD45-FITC (green fluorescence) vs. SS

Histogram 2: CD34-PE (orange-red fluorescence) vs. SS

Histogram 3: CD45-FITC (green fluorescence) vs. SS

Histogram 4: FSC vs. SSC

Histogram 5: CD45-FITC (green fluorescence) vs. CD34-PE (orange-red fluorescence)

Histogram 6: FSC vs. SSC

Histogram 7: Time vs. counting-bead fluorescence (for Group A instruments) or time vs. FSC (for Group B instruments)

Histogram 8: 7-AAD vs. SSC (Group A instruments with 4 FL detectors) or 7-AAD vs. SSC (Group B instruments). On Group A instruments with only 3 FL detectors, replace the 620-nm red fluorescence band-pass filter with a 675-nm band-pass filter for optimal detection of 7-AAD.

### 24.2.2 Creation of Gating Regions

In the following steps, specific terminology for regions and gates are given parenthetically for different instruments. For example, region A is for Group A instruments, and region R1 and gate G1 (see below) are for Group B instruments. See Figures 2 and 3.

Histogram 1 (leukocyte gate): Display all events. Draw a rectangular gate (A; R1) to include all CD45dim to CD45bright events and to exclude debris, platelets, and unlysed RBC, which are all CD45<sup>+</sup>.

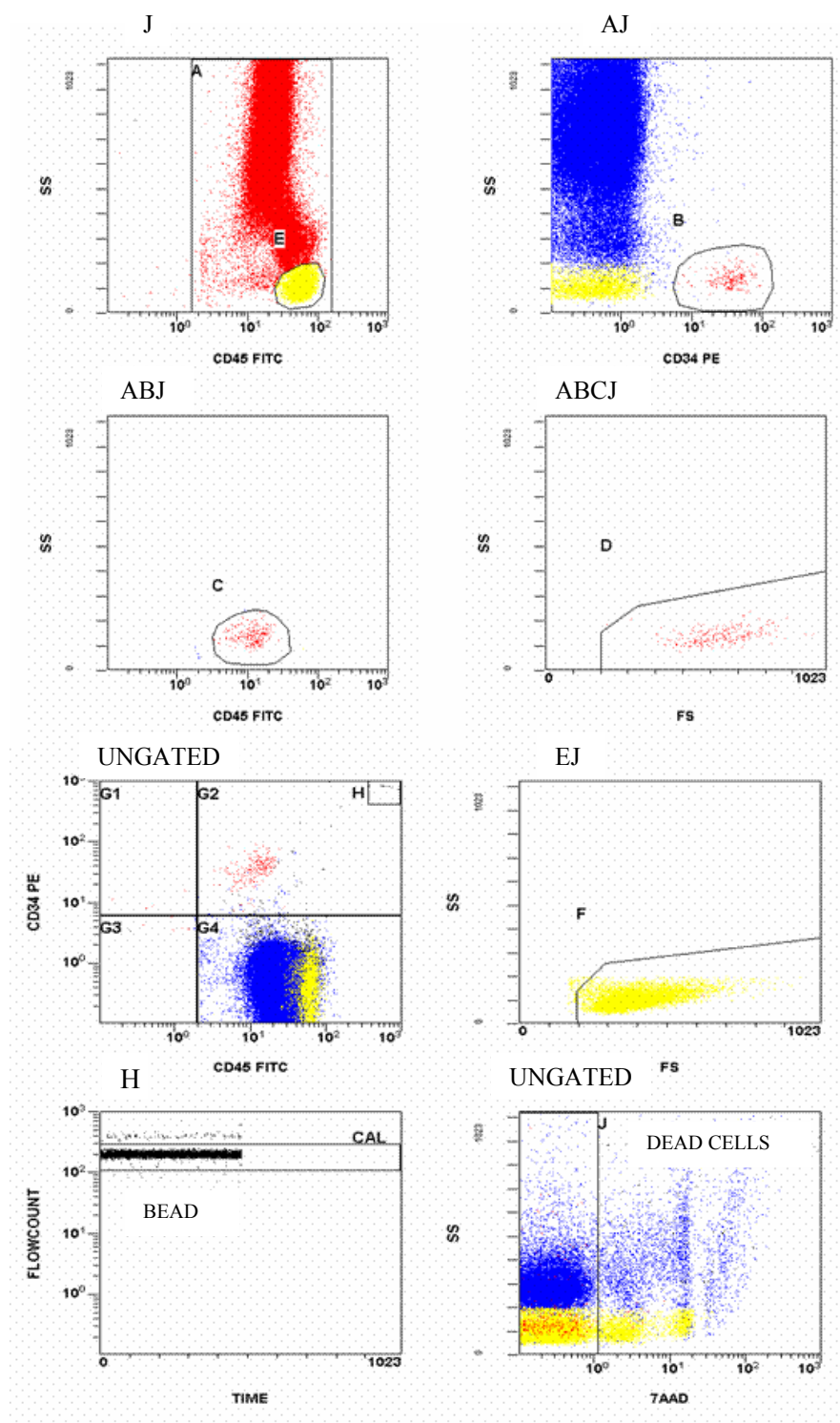


**Figure 2. Example of CD34<sup>+</sup> Cell Enumeration With 7-AAD Dead-Cell Exclusion for a Group A Flow Cytometer Equipped With Four Fluorescence Detectors.** Data are shown from a prediluted apheresis sample containing 12% dead cells (i.e., 7-AAD<sup>+</sup>) and 200 viable CD34<sup>+</sup> cells/ $\mu$ L. The cell dilution factor was 1/10. In this example, 1120 viable CD34<sup>+</sup> cells were gated in region D and 4020 singlet beads were gated in region H. The assayed bead concentration was 1004/ $\mu$ L.

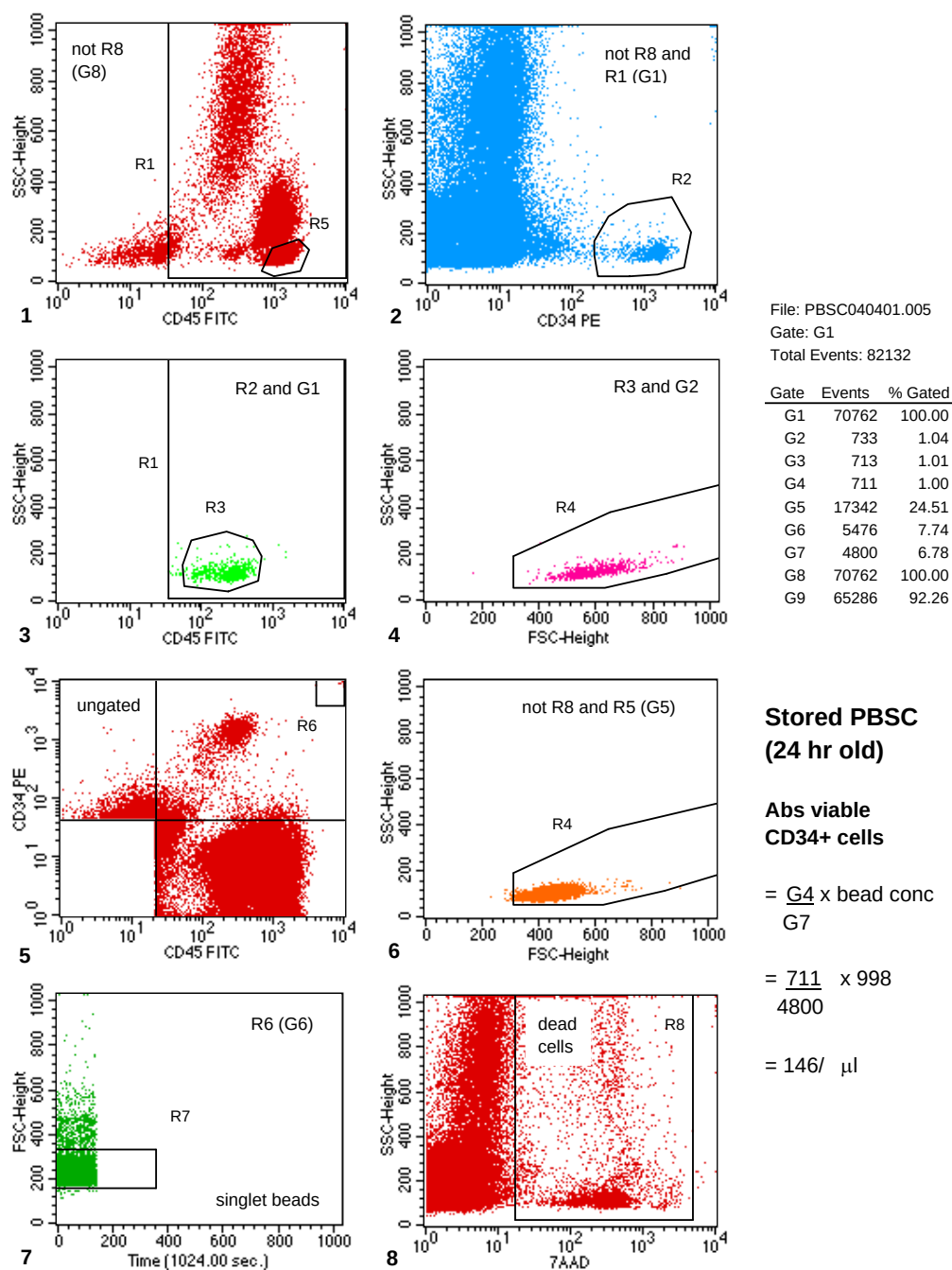


**Figure 3. Example of CD34<sup>+</sup> Cell Enumeration With 7-AAD Dead-Cell Exclusion for a Group B Flow Cytometer.** Data are shown from an apheresis specimen that had been stored overnight at room temperature. Sample (diluted 1/10 before staining) contained approximately 10% dead cells (i.e., 7-AAD<sup>+</sup> gated in R8) and 146 viable CD34<sup>+</sup> cells/uL. In this example, 711 viable CD34<sup>+</sup> cells were identified in gate 4 and 4800 singlet beads were enumerated in gate 7. The assayed bead concentration was 998/uL.

**NOTE:** For Group A instruments: By ensuring that the right edge of region A does not extend further than the brightest CD45<sup>+</sup> cells, the counting beads (in the top right-hand corner of histogram 1) can be excluded from region A. For Group B instruments: The counting beads are found in the brightest green, orange-red, and red channels and also exhibit very high side scatter. Since the gate statistics (see Figure 3) are obtained from events gated in R1 (displayed on histogram 2), it must be ensured that all the CD45<sup>+</sup>

events, as well as all the counting beads, are gated in R1 by including the highest green fluorescence and SSC channels.

Histogram 2 (total CD34<sup>+</sup> gate): Display events from the leukocyte gate (i.e., gated on A; gate G1 = R1). Draw an amorphous polygon (nonrectangular) region (B; R2) to include all CD34<sup>+</sup> events.

Histogram 3 (CD34<sup>+</sup> blast gate): Display events that fulfill the criteria of both above gates (i.e., regions A and B, gated on AB; regions R1 and R2, G2 = R2 and G1). Draw an amorphous polygon region (C; R3) to include only those events that form a cluster with low to intermediate SS and CD45<sup>dim</sup> expression.

Histogram 4 (lymph-blast gate): Display events that fulfill the criteria of all three above gates (i.e., regions A, B, and C, gated on ABC; regions R1, R2, and R3, G3 = R3 and G2). Draw an amorphous polygon region (D; R4) to include only those events that form a cluster with low to intermediate SSC and low to high FSC. On Group B instruments, set logical gate G4 = R4 and G3.

**NOTE:** The lymph-blast gate serves to exclude platelets and debris that may show weak nonspecific binding of CD34 and CD45 mAb. Its lower FSC boundary is verified in histogram 6 (see below).

Histogram 5: Display ungated data. Draw a quad-stat region in order to establish the lower limit of CD45 expression by the CD34<sup>+</sup> events. On histogram 5, draw a small rectangular region H (R6) to include the brightest events that fall in the highest green and orange-red channels. Although not readily visible, all counting beads (e.g., singlets and aggregates) are contained in this region.

Histogram 6 (duplicate lymph-blast gate): On histogram 1, draw an amorphous polygon region (E; R5) on the lymphocytes (CD45<sup>bright</sup>, SSC<sup>low</sup>) to create a lymphocyte gate. Display the events from this region in histogram 6 (i.e., gated on E; G5 = R5). Then:

*For Group A instruments:* draw an amorphous polygon region F in histogram 6 that is similar to region D in histogram 4, creating the duplicate lymph-blast gate. Adjust region F on histogram 6 so that only viable lymphocytes from region E are included. Once region F has been optimized, place region D in histogram 4 similarly.

*For Group B instruments:* Copy region R4 from histogram 4 into histogram 6 (creating the duplicate lymph-blast gate), and adjust the position of the duplicate so that only viable lymphocytes from region R5 are included. The original region R4 on histogram 4 will automatically move to the same position on histogram 4.

**NOTE:** This step establishes the minimum FSC and SSC ranges for the lymph-blast gate (F; R4) in histogram 6.

Histogram 7 (bead gate):

*For Group A instruments:* Set a rectangular region (G) to include only single-bead events, as per the counting beads manufacturer's recommendations. Adjust counting-bead (red fluorescence) high voltage to ensure that both singlet and doublet bead populations are visible. On the Group A instruments, gate on region H from histogram 5 and define region G as a CAL region to allow automatic calculation of absolute numbers of CD34<sup>+</sup> cells.

*For Group B instruments:* Display events from R6 on histogram 7 (time vs. FSC). If beads are not visible, lower FS threshold until singlet beads are clearly visible. Set region R7 to include only singlet beads. Set logical gate G7 = R6 and R7.

Histogram 8 (exclusion of dead cells):

*For Group A instruments:* Draw a rectangular region (J) to include only living (i.e., 7-AAD<sup>-</sup>) cells, and display data from region J only in histograms 1 to 6. A summary of the resulting logical gates is shown below.

*For Group B instruments:* Draw a rectangular region (R8) on histogram 8 that includes the dead cells (7-AAD<sup>+</sup>) but excludes the counting beads that are present in the highest SSC and FL3 channels). Exclude dead cells from further analysis by setting logical gates as shown below.

**Table 2. Summary of Instrument-Specific Logical Gates Using Dead Cell Exclusion**

| Histogram | Group A       | Group B              |
|-----------|---------------|----------------------|
| 1         | Gated on J    | G8 = not R8          |
| 2         | Gated on AJ   | G1 = (not R8) and R1 |
| 3         | Gated on ABJ  | G2 = R2 and G1       |
| 4         | Gated on ABCJ | G3 = R3 and G2       |
| 5         | Ungated       | Ungated              |
| 6         | Gated on EJ   | G5 = (not R8) and R5 |
| 7         | Gated on H    | G6 = R6              |
| 8         | Ungated       | Ungated              |

### 24.2.3 Data Acquisition

Acquire a minimum of 100 CD34<sup>+</sup> events (region D; G4 = R4 and G3) from the first CD34 tube. If a detection level of 0.1% is sufficient, stop at 50 000 CD45<sup>+</sup> events (region A; R1). Repeat this step for the second CD34 tube.

**NOTE:** The total number of events acquired is dependent on the desired sensitivity (e.g., a level of 0.1% implies the detection of 100 CD34<sup>+</sup> cells among a total of 100 000 CD45<sup>+</sup> cells) in the two tubes analyzed.

### 24.2.4 Data Analysis

If the total number of events in region D (G4 = R4 and G3) of the two CD34 tubes is less than 100, calculate the absolute number of CD34<sup>+</sup> cells per  $\mu\text{L}$  as follows (for software that does not calculate the absolute CD34<sup>+</sup> cell number automatically):

$$\text{CD34}^+ \text{ cells}/\mu\text{L} = \frac{\# \text{CD34}^+ \text{ cells} \times B \times \text{DF}}{\# \text{beads}}$$

In this equation, the number (#) of CD34<sup>+</sup> cells is determined from region D (G4 = R4 and G3), the number of singlet beads is determined from region G (G7), *B* is bead concentration (specified per lot), and DF is the sample dilution factor. Average the results of the two tubes.

Caution should be used in the case of samples with an absolute number of CD34<sup>+</sup> events in region D (G4 = R4 and G3) <100, as the precision of the assay will be reduced by the low number of cells enumerated.

**NOTE:** On Group B instruments, the counting beads are present (but not visible) in the top right corner of region R1 and must be removed from the total number of events present in R1 for accurate calculation of percent CD34<sup>+</sup> cells. This can be accomplished by manually subtracting the number of total beads (i.e.,

singlets and doublets) in region R6 (G6) from the number of events in R1 (G1), or by setting an additional logical gate (e.g., G9 = not R6 and R1), as shown in Figure 3.

The basic protocol may also be performed using commercially available absolute counting tubes instead of microspheres. When such prealiquoted tubes are used, the manufacturer's instructions must be followed for making the absolute cell count calculations. In this and other settings, when the counting beads are very small, a threshold cannot be set on forward scatter, making it necessary to set it on a fluorescence parameter. An advantage of using prealiquoted beads is that it eliminates the requirement to carefully suspend and pipette the counting beads. However, accurate pipetting is still required if any predilution of the sample is required, as well as for the delivery of the sample into the tube. Note that some sample types (e.g., blood or cord blood) prepared by lyse/no-wash sample processing, may contain a large amount of debris. By setting a gate to exclude the CD34<sup>+</sup>,45<sup>-</sup> events on histogram 5 before data acquisition, a major portion of the debris can be excluded from the list-mode file. An example of a data file acquired using this modification is shown in Figure 3.

## 25 Data Storage

The possibility of patients contesting therapeutic implications derived in part from CD34<sup>+</sup> cell enumerations in blood or apheresis products requires that the laboratory is able to verify the process used in arriving at the reported test results.

### 25.1 Information Storage

Store list-mode data for all specimens analyzed. This policy allows for a comprehensive reanalysis of the raw data. At a minimum, retain hard copies of the eight bivariate histograms showing the analyses for CD34<sup>+</sup> cell enumeration of each sample.

Retain all primary files, worksheets, and report forms for two years or as required by state or local regulation, whichever is longer. Data can be stored electronically (see above); backup of the data shall be in place to prevent loss of patient data in case of hardware or software failure.<sup>44</sup> Disposal after the retention period is at the discretion of the laboratory director.

QA/QC data files should include all parameters, analysis regions, and analytical results used to verify system (instrument and method) performance.

### 25.2 Types of Data Storage

#### 25.2.1 Paper Hard Copy

If data are stored as paper hard copy, all histograms and analysis regions used in arriving at the reported test results must be recorded on the hard copy of the relevant data plot. A unique specimen identifier should also be recorded with each data plot.

#### 25.2.2 Archival Files

A variety of data storage media may be used, depending on the particular systems available in the laboratory, including floppy disks, removable hard disks, optical disks, and magnetic tape.

### 25.3 Duration of Data Storage

Patient data should be kept for a minimum of two years, or as required by state and federal (national) law, whichever is longer. After that, it is the responsibility of the laboratory director to determine whether to save or discard the data. QA/QC data should be kept for a minimum of two years.

Data may be stored for as long thereafter as the laboratory director considers necessary, providing storage space is available.

## **26 Data Reporting and Interpretation**

### **26.1 Worksheet**

A standard laboratory worksheet should be developed for reporting results of CD34<sup>+</sup> cell enumeration assays.

Report all data in terms of CD designation with a short description of what that designation means (see Section 11.1).

### **26.2 Supervisory Check**

Recording of laboratory data and calculations should be checked by supervisory personnel, and authorized.

### **26.3 Review of Data Displays**

Data displays showing discriminator/threshold setting during data acquisition, the number of events acquired, the light scatter patterns and antibody staining profiles, and all analytical gates and fluorescence cursor settings used to arrive at the test result should be reviewed by a laboratory professional when interpreting the data.

### **26.4 Reporting of Data**

All data should be reported as absolute numbers of viable CD34<sup>+</sup> cells. To give an indication of overall sample quality, total sample viability should also be provided. This is of particular importance in cord blood and any other sample that has been stored for more than four hours. Apheresis products stored overnight before further processing should be retested and have viability assessed immediately before cryopreservation. For apheresis products and cord blood units, calculate the number of CD34<sup>+</sup> cells per kilogram of patient body weight in order to enable the transplant physician to judge whether the collected number of CD34<sup>+</sup> cells is sufficient to perform the stem cell transplant. This calculation requires the absolute CD34<sup>+</sup> cell count, the volume of the apheresis pack or cord blood unit, and the patient's body weight.

### **26.5 Interpretation of Data and Reference Intervals**

Data interpretation should be made using an established laboratory or clinical target. Many laboratories consider the range of 5 to 10 CD34<sup>+</sup> cells/ $\mu$ L as a threshold below which the patient will not undergo an apheresis procedure. If the WBC concentration is less than  $0.7 \times 10^9$  cells/L, it may be inappropriate to perform apheresis, since at such low blood counts it can be problematic to accurately determine the mononuclear cell interface during cell processing. For apheresis products, the target range of CD34<sup>+</sup> cells is expressed per kilogram of patient body weight, and is usually in the range of 2 to  $5 \times 10^6$  CD34<sup>+</sup> cells/kg for a single transplant procedure.

### **26.6 Notation of Out-of-Range Control Samples**

If the results of analysis of positive method control samples are outside the laboratory reference interval and no reason has been determined, results on patient specimens prepared at the same time are potentially suspect, and this should be noted on the test report.

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## Appendix A. Instrument Setup and Quality Control of Instrument Performance

### A1. Introduction

The verification of the reproducibility of instrument performance is an essential element of the daily quality assurance in FCM. Instrument performance must be monitored under the same conditions as those used to run test samples. Quality control of instrument performance therefore involves two procedures:<sup>1</sup>

- **Initial instrument setup**—identification of appropriate conditions for analysis of test samples and establishment of acceptance values for materials used in daily instrument monitoring. This procedure is carried out at relatively long intervals (e.g., once every six months) by qualified service personnel, and includes examination of the efficiency and performance of the laser tube, optical filters, log and linear amplifiers, and photomultiplier tubes (PMT) of all types of flow cytometers. These procedures also include calibration of the optical alignment of flow cytometers equipped with a cuvette to ensure that the brightest and tightest peaks are produced in all parameters. Most clinically used flow cytometers are of the “stream-in-cuvette” type. However, optical alignment of the larger flow cytometers with sorting facilities, which are equipped with a nozzle (“stream-in-air” type), must be calibrated daily by the instrument operators due to its relative instability.
- **(Daily) monitoring of instrument performance**—This group of procedures consists of frequent monitoring of instrument performance by the operators to identify both immediate and potential problems (i.e., with each “cold start” of the instrument). After having established the proper instrument settings, channels and coefficients of variation (CV) for important parameters using suitable reference microbeads are recorded (see below), and the results of the day are then compared with established tolerance limits. Trends and variations can be noted with the use of Levy-Jennings style charts that allow visual inspection of longitudinal data for monitoring instrument performance for precision and trends. If any value falls outside the tolerance limits, monitoring should be repeated and instrument settings adjusted if problems persist.

Many of the issues involved in instrument QC for immunophenotypic analysis of hematologic malignancies are similar to those encountered in analysis of non-neoplastic WBC. However, the issues of sensitivity and color compensation take on particular importance. Neoplastic WBC frequently exhibit aberrant intensities of antigen expression and/or aberrant patterns of antigen coexpression. If instrument sensitivity is inadequate, dim antigen expression may not be detected. If color compensation is excessive, a population expressing one antigen may be incorrectly considered to lack another. Therefore, it is particularly important to validate instrument performance by using samples with characteristics as similar as possible to those of the test samples.

Table A1 summarizes the various tasks involved in instrument quality assurance and the general properties of samples that are necessary in performing each task. For each quality assurance (QA) function, the corresponding section in this appendix is specified.

## Appendix A. (Continued)

**Table A1. Quality Assurance (QA) Overview**

| <b>QA Function</b>                                                   | <b>Why</b>                                                                                                                                                                       | <b>When</b>                                                                                                                                     | <b>What (Sample Properties)</b>                                                                                                                                                                                                                                                                                          |
|----------------------------------------------------------------------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-------------------------------------------------------------------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| <b>Initial Instrument Setup</b>                                      | <b>Optimize Analysis Conditions</b>                                                                                                                                              | <b>New Test or Instrument Conditions</b>                                                                                                        | <b>Various (see below)</b>                                                                                                                                                                                                                                                                                               |
| Optical alignment (A2.1)                                             | Maximize intensity, minimize variability.                                                                                                                                        | New instrument, major maintenance or repair                                                                                                     | Homogeneous signal in all channels used for specimen analysis; long-term stability reference beads                                                                                                                                                                                                                       |
| Position of the “window of analysis” in “sample space” (A2.2)        | Adjust the amplifier gain or PMT settings for each parameter such that the routine specimens for that application appear in the range of histogram channels of these parameters. | New instrument, major maintenance or repair<br>Introduction of test; after any significant changes in specimen preparation or staining protocol | “Normal” specimen prepared using the laboratory’s standard test protocol or reference beads with predefined acceptance values established under initial optimal test-specific instrument settings                                                                                                                        |
| Setting electronic compensation for spectral overlap (A2.3)          | Verify that after labeling of two or more different fluorochromes, multiple-labeled cells can be accurately distinguished from single-labeled cells.                             | New instrument, major maintenance or repair<br>Each time FL PMT settings are changed                                                            | Cells labeled with reagents giving dim-to-bright intensities conjugated to all relevant fluorochromes (e.g., CD8-FITC, CD8-PE) or fluorochrome-conjugated or labeled beads with acceptance values confirmed by labeled cells and with intensities and spectral characteristics similar to bright antibody-positive cells |
| Linearity validation (A3)                                            | Verify linear detector response.                                                                                                                                                 | New instrument, major maintenance or repair                                                                                                     | Series of beads with at least four different predefined levels of FI, including nonfluorescent beads                                                                                                                                                                                                                     |
| <b>Instrument performance monitoring</b>                             | <b>Ensure acceptable performance.</b>                                                                                                                                            | <b>Whenever samples are run</b>                                                                                                                 | <b>Stable; having established acceptance values</b>                                                                                                                                                                                                                                                                      |
| Monitor intensity performance and fluorescence resolution (A4)       | Verify reproducible intensity measurements and acceptable sensitivity.                                                                                                           | Whenever samples are run or instrument malfunction is suspected                                                                                 | Reference beads with predefined acceptance values established under optimal test-specific instrument settings                                                                                                                                                                                                            |
| Calibration of the instrument’s response to fluorescent signals (A3) | Standardize measured fluorescence intensity (FI).                                                                                                                                | Whenever quantitative flow cytometry (QFCM) is performed                                                                                        | Bead(s) or cell(s) with predefined fluorescence intensity (intensities)                                                                                                                                                                                                                                                  |

## **Appendix A. (Continued)**

### **A2. Initial Instrument Setup**

#### **A2.1 Optimization of Optical Alignment**

Proper alignment of the optical components of the flow cytometer (e.g., laser, flow cell, focusing lenses, collecting lenses, photodetectors) should be established using the manufacturer's recommended alignment materials and procedures. Alignment particles are typically uniform latex particles incorporating a fluorescent dye; other materials may also be recommended by the manufacturer.

The goal in optimizing alignment is to simultaneously achieve maximum signal intensity (i.e., maximum mean channel number) and minimum signal variability (i.e., minimum coefficient of variation [CV]) for all parameters that will be used to analyze test specimens, including forward and sideward light scatter.

For instruments with fixed optical systems, such as most current bench-top flow cytometers where daily alignment is not carried out by the user, alignment should be verified using manufacturer-recommended particles to determine whether instrument performance meets specifications.

In some cases, manufacturers provide expected values for their particles when used, for example, with specific instruments, instrument settings, and filters. In other cases, the laboratory must determine optimum settings for its own instrument-alignment particle combination and establish its own expected values.

#### **A2.2 Positioning of the “Window of Analysis” in “Sample Space”**

Dependent on the application, the amplifier gain or PMT settings for each parameter require adjustment, such that the routine specimens for that application appear in the range of histogram channels of these parameters (i.e., the “window of analysis”). Changing the amplifier gain or PMT settings repositions this window in “sample space” (i.e., the fluorescent and light scatter characteristics of the sample that are independent of the instrument). For positioning of the fluorescence (FL) windows of analysis, it is recommended that, for each type of assay, unstained cells are placed in the first of four log decades, above the level of instrument noise. Depending on the fluorochrome, autofluorescence of unstained cells may be greater than that of unstained beads at the recommended PMT setting.

This approach has the following advantages:

- (1) The observation that unstained beads often fall in a lower range of channels than unstained cells ensures that the instrument's noise does not interfere with the result of the immunophenotyping assay.
- (2) The positioning of single-stained and unstained cells slightly away from the axes of the bivariate FL allows appropriate adjustment of electronic compensation for spectral overlap.

#### **A2.3 Setting Electronic Compensation for Spectral Overlap (“Color Compensation”)**

Compensation is the process of correcting for spectral overlap of one fluorochrome into the filter window used to monitor another fluorochrome (see Figure A1). In most instruments used clinically, this correction is done by adjusting the electronic compensation circuits on the flow cytometer to place populations not expected to be dual-positive for two fluorochromes into orthogonal fluorescence quadrants, with no overlap into the double-positive quadrant. At the same time, avoiding overcompensation is essential because overcompensation may cause incorrect classification of dual-positive cells as single positive. The procedure may be performed manually; alternatively, software may perform the spectral compensation automatically (see below). Spectral overlap of fluorochromes must be corrected for to

## Appendix A. (Continued)

ensure the accurate enumeration of cells labeled with multiple fluorochromes. Accurate compensation is accomplished by staining a control blood sample containing mutually exclusive populations of cells, carrying the same fluorochrome as used in the experimental samples, and by processing this control sample in an identical way to the experimental samples. (The best method is to singly stain cells using each of the antibodies in the experiment. This way, the laboratorian ensures that the spectral characteristics for each antibody are correctly represented.) In this respect, the use of blank and externally labeled beads may be inferior to the use of cells, because the spectral characteristics of fluorochromes directly bound to beads are slightly different from those of fluorochromes bound to antibodies. Especially on instruments using analog log amplification, setting color compensation using the FL signal of labeled beads relative to that of blank beads results in slight overcompensation, because blank beads have dimmer FL signals than unstained cells, due to the autofluorescence of the latter. If standardization or calibration particles (microbeads) are used to set compensation, appropriate color compensation can be confirmed by using lymphocytes labeled with FITC- and PE-labeled monoclonal antibodies and a third-color- or fourth-color-labeled monoclonal antibody for three-color or four-color panels, respectively.

The spectral characteristics of most fluorochromes (i.e., FITC, PE, PerCP, APC) will be similar when conjugated to different antibodies. Exceptions to this rule are the tandem fluorochromes (see below). The electronic correction of spectral overlap is a function of the PMT settings involved. Therefore, each time the settings of an FL PMT are changed, compensation settings must be verified and corrected. In contrast, changing FSC photodiode or SSC PMT settings does not affect compensation.

In the setting of multicolor analyses, visualization of compensated data may often result in unexpected distributions—mainly the appearance of large numbers of events on the axis, but also the inability to reduce the range of compensated data to “autofluorescence” levels. In such situations, it is impossible to achieve appropriate color compensation using standard visual graphical interfaces (i.e., bivariate histograms using log scales); manual adjustments of compensation will always result in overcompensation. Therefore, computer-assisted adjustment of compensation is required, as well as careful gating and experimental design to discriminate between positive and negative events. At a minimum, such software should provide median statistics or a graphical representation of the median as a function of intensity of positive and negative populations during any user-controlled adjustment. Alternatively, all adjustments are made automatically without user intervention <sup>2,3</sup>

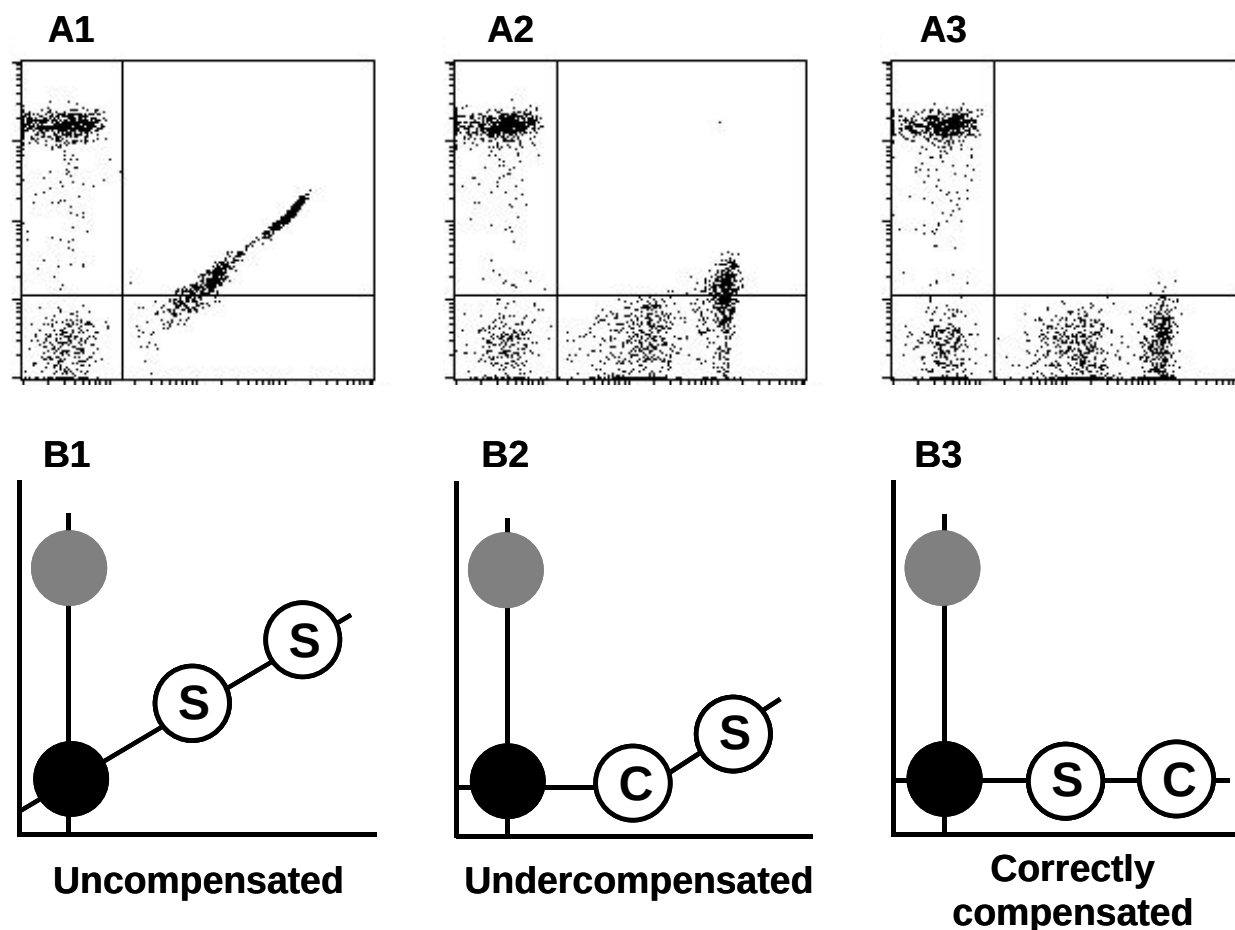
**Appendix A. (Continued)**

**Figure A1.** Histograms A1 to A3 and their respective schematic depictions, B1 to B3, illustrate uncompensated, undercompensated, and correctly compensated two-color displays, respectively. The open circles labeled “S” represent the cell population for which accurate color compensation is desired. The open circles labeled “C” represent the compensation controls used to adjust color compensation settings. If the intensity of the control does not exceed the fluorescence intensity of the sample analyzed, that sample will be undercompensated (A2, B2). If the intensity of the control exceeds the fluorescence intensity of the sample analyzed, the sample is less likely to be undercompensated (A3, B3). **NOTE:** In panels A3 and B3, the median fluorescence (y-axis) of the unstained, compensated particles and stained cells will be approximately equal.

With the expanding parameter capability in FCM, newly developed fluorochromes now include the so-called tandem dyes, whose design is based on the principle of fluorescence resonant energy transfer (FRET). In FRET, the donor dye absorbs energy from the illuminating source and transfers that energy to a partner or acceptor dye without emitting photons. The partner dye then emits photons at its own characteristic wavelength, permitting excitation of several colors from a single laser. Nevertheless, for some conjugates, FRET is incomplete, and compensation must be used to suppress the effect of photons “leaking” at the donor dye’s usual emission wavelength. “Good” tandem dyes require minimal compensation (1 to 5%) of the donor dye parameter, but some other tandems may require  $\geq 10\%$  compensation, jeopardizing the detection of weakly expressed antigens by masking fluorescence. This problem is aggravated by the fact that quite often, the very same fluorochrome as is the donor dye in the tandem (i.e., phycoerythrin [PE]) is selected for measurement of dimly expressed antigens because

## Appendix A. (Continued)

of the brightness of PE. To avoid this “loss of sensitivity,” staining panels should be constructed selecting those antigens whose expressions are mutually exclusive for the single-color donor dye and the tandems, respectively. Figure A2 illustrates five commonly used fluorochromes with measurable “leakage” into other fluorochromes. The significant leakage of the tandem fluorochrome PC7 (PE-Cy7) into the emission wavelength of PE should be noted. Importantly, spectral overlap is not unique to tandem dyes; in particular, FITC has significant overlap into PE, and PE into ECD (PE/Texas Red tandem fluorochrome). Fluorochrome/antibody combinations should always take these overlaps into account when designing panels.

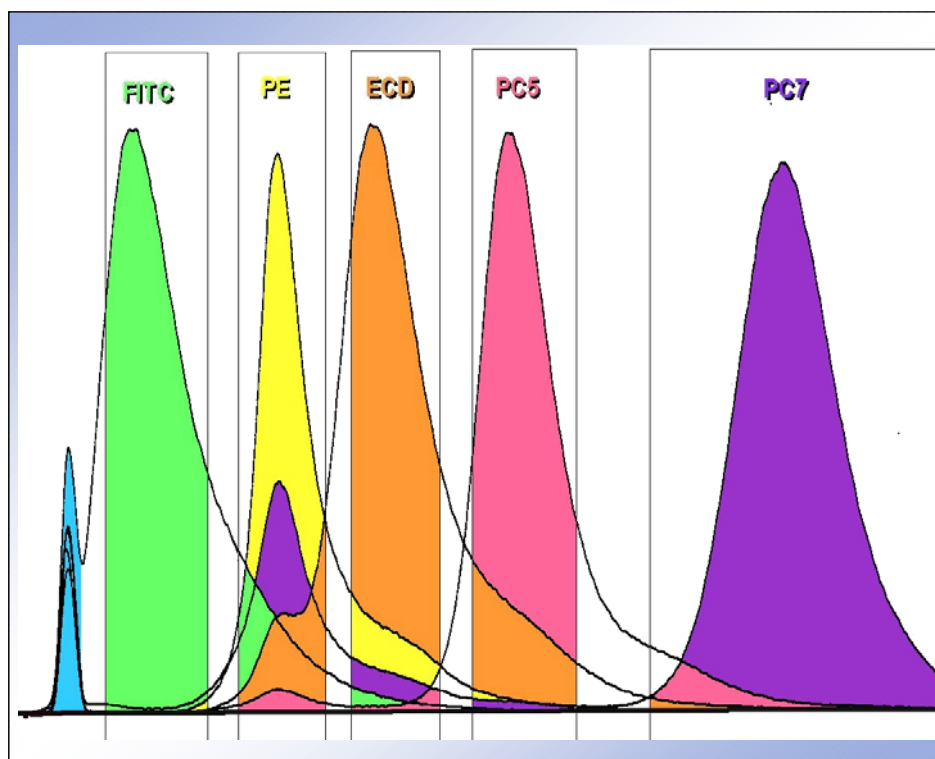


**Figure A2. Spectrum of Wavelength Emission of Various Fluorochromes.** To simplify the display, the color intensities of the various fluorochromes have been normalized. PC5=PE-Cy5. PC7=PE-Cy7.

### A3. Linearity Validation and Calibration of the Instrument’s Response to FL Signals

Since fluorescence intensity is used to distinguish “positive” from “negative” cells in immunophenotyping assays, it is important to ensure reproducibility and stability of FL intensity assessments over time. This QA procedure is done by calibrating the flow cytometer using a series of beads with at least four different predefined levels of FL intensity, including nonfluorescent beads.<sup>4,5</sup> In the most current edition of CLSI/NCCLS document I/LA24—*Fluorescence Calibration and Quantitative Measurement of Fluorescence Intensity*, the basic principles and laboratory practices involved in quantitative flow cytometry (QFCM) are outlined. This guideline allows the generation of a linear calibration curve for each fluorescence detector and for a given flow cytometric assay. In this context, it is important to differentiate between a linear detector response and the linear range of a specific assay. However, the principles are applicable to both situations.<sup>6,7</sup>

## Appendix A. (Continued)

QFCM is the measurement of FL intensity in terms of relative fluorescence intensities (RFI) or molecular equivalents of soluble fluorochrome (MESF).<sup>7</sup> For each fluorochrome, a calibration standard is constructed to obtain a standardized determination of FL intensity. A reference method is described for constructing an FI calibration curve and evaluation of instrument performance parameters.<sup>7</sup> The proper construction and interpretation of a calibration curve for QFC requires the selection of appropriate standards,<sup>5</sup> as well as the correct transformation of units of FL intensity for reporting results.<sup>7</sup>

There are five recognized approaches for generating a standard calibration regression line. First, the simplest approach consists of internally dyed microspheres that have been assigned relative FL intensity (RFI) values, as these standards do not spectrally match the fluorochromes that are routinely used for QFCM (e.g., FITC, PE, APC). Second—and in contrast to the first approach—MESF microbead standards do spectrally match these fluorochromes. In this context, it is relevant that that National Institute of Standards and Technology (NIST) has developed a fluorescein reference solution (SRM 1932) for use in conjunction with the FITC-labeled microsphere reference material RM 8640.<sup>8</sup> Third, a modification of this approach is based on commercially available PE-labeled microsphere calibrators used in conjunction with mAb that have been labeled 1:1 with PE (i.e., 1 PE molecule to 1 mAb molecule). The calibration regression line constructed in this way allows the expression of FL intensity in terms of numbers of mAb molecules bound per cell (ABC).<sup>9-11</sup> Fourth, Giorgi et al combined an RFI-based calibration regression line with a biological calibrator (i.e., CD4<sup>+</sup> T-cells). As the number of CD4 molecules per T-cell is very stable (i.e., 50 000 per cell), this characteristic was used to derive the numbers of molecules of interest per cell (such as CD38) from the calibration curve.<sup>12</sup> In a further variation of this method, Davis et al used a known MESF microbead to standardize the measurement of CD64 expression for the diagnosis of sepsis.<sup>13</sup> Fifth, Poncelet et al developed biological calibrators to construct a calibration regression line for indirect immunofluorescence stainings.<sup>14</sup>

Most of these systems allow the user to generate calibration regression lines and to derive from these lines instrument performance parameters for each FL channel. Five of these parameters have been defined: linearity or average residual percent, slope or coefficient of response, zero channel, maximum channel, and detection threshold (see Table A2). The average absolute percent residual gives the average percent disagreement between the assigned and best-fit dose values for all data points, and is the simplest aggregate parameter of overall linearity. The slope or coefficient of response is a measure of the number of channels per decade on a log scale. Extrapolating the calibration curve to the zero and maximum channels can be used to define a common window of analysis (see above). The use of a consistent window of analysis may facilitate quality assurance in longitudinal and multicenter studies. The detection threshold is complicated both from the standpoint of instrumentation and the biological assay.<sup>15-17</sup>

In summary, instrument performance parameters provide the most objective way to validate instrument performance and compare FL measurements across time, instruments, and platforms. Thus, monitoring of these parameters provides the most comprehensive approach to instrument QC and assurance of FL measurement accuracy.

**Appendix A. (Continued)****Table A2. Instrument Performance Parameters for Measurements of Fluorescence<sup>7</sup>**

| <b>Average residual percent</b> |                                                                                                                                                                                                                                                                                        |
|---------------------------------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| <i>Definition</i>               | The absolute average percent that the calibration regression line between a series of calibration beads labeled with different FL signals of predefined intensities, and the instrument's response (in histogram channels) deviates from the actual data points.                       |
| <i>Significance</i>             | <i>Indicates:</i> <ul style="list-style-type: none"> <li>the linearity or goodness of fit of the calibration line; and</li> <li>the confidence of the instrument's response across the FL intensity range.</li> </ul>                                                                  |
| <i>Tolerance zone</i>           | (arbitrary) < 5%                                                                                                                                                                                                                                                                       |
| <b>Detection threshold</b>      |                                                                                                                                                                                                                                                                                        |
| <i>Definition</i>               | Value, in arbitrary units of FL intensity, of the histogram channel at the right-hand foot of the peak of nonfluorescent standards.                                                                                                                                                    |
| <i>Significance</i>             | <i>Indicates:</i><br>the lowest level of fluorochrome that can be detected by the instrument.                                                                                                                                                                                          |
| <i>Tolerance zone</i>           | <i>Defined by the user</i>                                                                                                                                                                                                                                                             |
| <b>Coefficient of response</b>  |                                                                                                                                                                                                                                                                                        |
| <i>Definition</i>               | The slope of the calibration regression line (see under "Average residual percent") calculated using a linear scale. Indicates a theoretical value for the number of histogram channels per decade on a log scale (e.g., four-log decade and 256-channel resolution: $256/4 = 64.0$ ). |
| <i>Significance</i>             | <i>Indicates:</i> <ul style="list-style-type: none"> <li>range of sample space covered by the window of analysis; and</li> <li>deviation from the theoretical value indicates suboptimal instrument performance.</li> </ul>                                                            |
| <i>Tolerance zone</i>           | (arbitrary) (instrument channel resolution ÷ # log decades) ± 5%                                                                                                                                                                                                                       |
| <b>Zero channel value</b>       |                                                                                                                                                                                                                                                                                        |
| <i>Definition</i>               | Intercept of the calibration line with the axis on which units of FL intensity have been plotted.                                                                                                                                                                                      |
| <i>Significance</i>             | <i>Indicates:</i><br>theoretical number of units of FL intensity corresponding with histogram channel 0; hence, it defines the left-hand boundary of the FL window of analysis.                                                                                                        |
| <i>Tolerance zone</i>           | <i>Defined by the user</i>                                                                                                                                                                                                                                                             |
| <b>Maximum channel value</b>    |                                                                                                                                                                                                                                                                                        |
| <i>Definition</i>               | Intercept of the calibration line with the axis on which units of FL intensity have been plotted.                                                                                                                                                                                      |
| <i>Significance</i>             | <i>Indicates:</i><br>theoretical number of units of FL intensity corresponding with the maximum histogram channel; hence, it defines the right-hand boundary of the FL window of analysis.                                                                                             |
| <i>Tolerance zone</i>           | <i>Defined by the user</i>                                                                                                                                                                                                                                                             |

## Appendix A. (Continued)

### A4. Monitoring of Instrument Performance

In order to maintain appropriate instrument settings over time, control or reference materials are measured under test-specific instrument settings and the values obtained are compared to the acceptance values previously established under conditions of optimal performance. Upon each “cold start” of the instrument, appropriate application-specific instrument settings must be obtained.

Two different strategies exist for this purpose:<sup>1</sup>

#### Strategy 1: Reestablish identical target channels (see Figure A3-1)

According to this strategy, daily adjustment of PMT settings of all FL parameters is performed to maintain a constant positioning of the window of analysis in sample space. For each FL parameter, reference beads are placed in identical target channels between runs. The daily adjustment of PMT settings implies that compensation settings must also be adjusted daily. The major manufacturers of flow cytometers have developed reagents and automated software for this purpose.

#### Strategy 2: Meeting “tolerance zones” (see Figure A3-2)

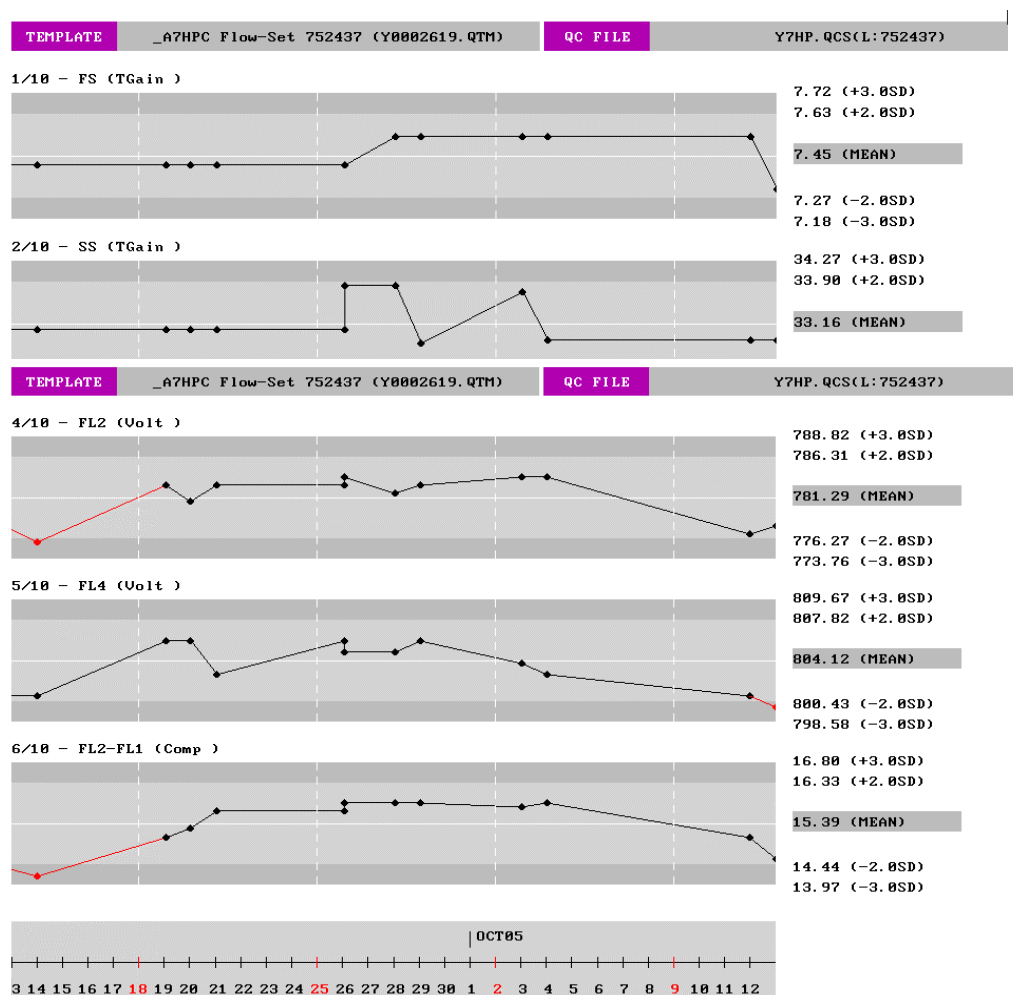
The appropriate positioning of the FL windows of analysis in sample space is monitored by verifying, using reference beads, whether or not measurement of reference beads after each “cold start” fall within predefined “tolerance zones.” For this purpose, the mean FL channels of the reference beads are plotted in Levy-Jennings charts. Adjustment of PMT settings and color compensation is only necessary if beads repeatedly fall outside these tolerance zones, which may be evaluated with Westgard’s multirules.<sup>18</sup> Once an instrument has been serviced, tolerance zones most likely require redefinition.

#### Comparison of both strategies:

Both approaches (i.e., reestablishing identical FL target channels, and meeting predefined FL tolerance zones over time) ensure that data collected over long periods of time will be meaningfully comparable, even if the instrument’s performance has been variable during that period, because the instrument’s responses to FL signals have been adjusted to perform relative to an independent benchmark. The advantage of Strategy 1 is its user-friendliness, thanks to the use of instrument-specific software. However, without this software, the approach would be very labor-intensive. Therefore, the manufacturer-defined procedures should be strictly followed. The advantages of Strategy 2 are twofold: (1) universal applicability (some instrument-specific systems based on Strategy 1 do not accommodate the use of specific fluorochromes); and (2) labor saving when multiple application-specific instrument settings have been verified at the same time (e.g., when multiple tandem conjugates are used, or when multiple sample preparation methods require specific instrument settings). If one of these settings is appropriate, it implies that the remaining settings are also adequate at that time point.

**NOTE:** In spite of proper alignment, calibration, and compensation control during system setup and QC, the quality of data generated is not guaranteed. Momentary fluidics variations, sample aggregations, or even occasional air bubbles passing through the flow cell can cause disturbances in the measured parameters. Looking at ungated data for shifts in light scatter characteristics helps to detect this problem before further analysis.

## Appendix A. (Continued)



Figures A3-1 to A3-3. Illustration of Two Strategies for Instrument Performance Monitoring

**Figure A3-1.** Strategy 1: detector gain or high voltage required to reproduce predefined mean channel number of a fluorescent setup bead for forward scatter (FS), side scatter (SC), FL2, and FL4. Compensation (FL2 to FL1) is shown in the lowest panel. The area with light-gray background indicates the “narrow-margin” tolerance zone (i.e., mean  $\pm 2$  SD), while the area with dark-gray background indicates the extension to a “wide-margin” tolerance zone (i.e., mean  $\pm 3$  SD).

## Appendix A. (Continued)

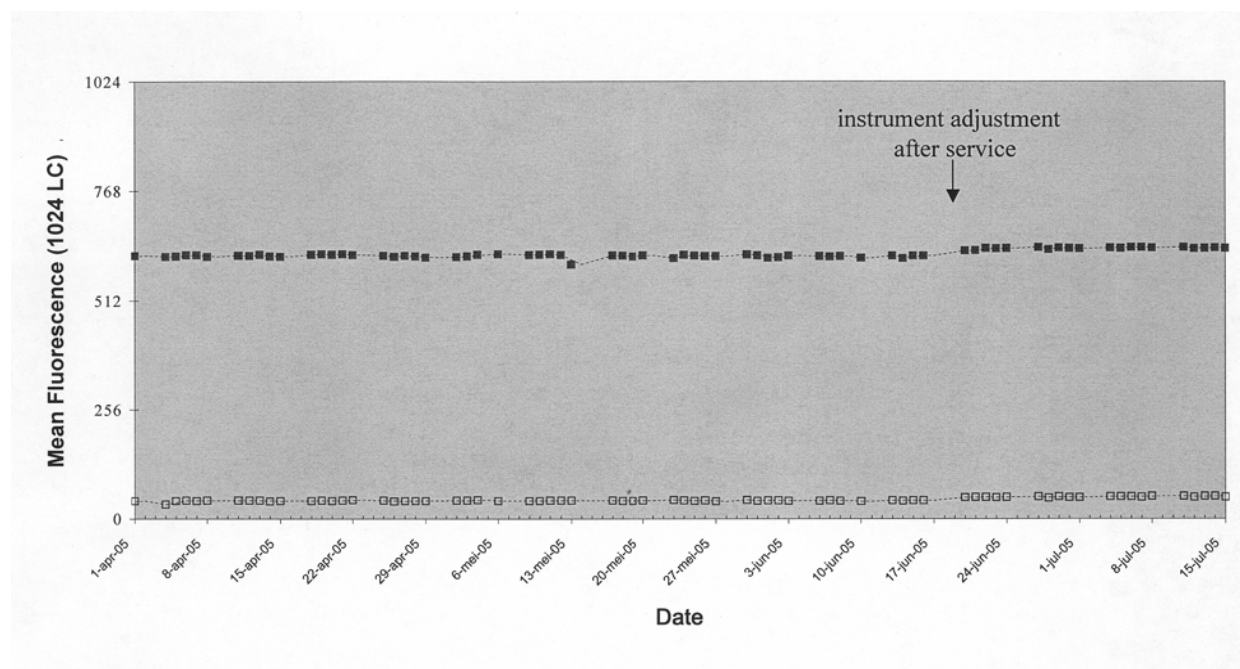


**Figure A3-2.** Strategy 2: mean fluorescence intensity of a blank bead (open squares) and fluorescent reference beads (closed squares) for an FL channel monitored under constant instrument conditions (i.e., constant FL detector high voltage/gain settings, compensation settings). The instrument was serviced on 19 June 2005, after which its settings were changed.

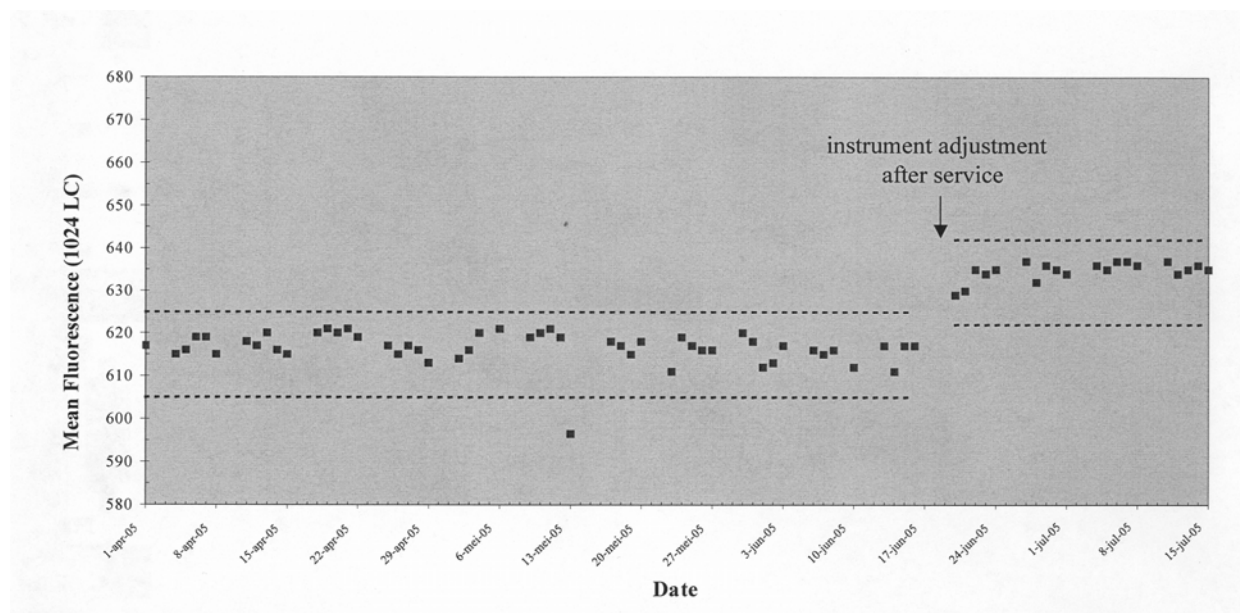


**Figure A3-3.** Magnification of the data on the fluorescent reference beads in Figure A3-2. The dashed lines indicate the established tolerance zones (mean  $\pm$  10 channels; 1024-channel linear histogram scale). The out-of-range value obtained on 13 May 2005 was related to temporary blockage of the instrument's flow cell, which was solved by simple rinsing.

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## Appendix B. Alternative Immunostaining Panels for CD4<sup>+</sup> T-cell Subset Enumeration

### B1. Introduction

Numerous combinations of reagents and immunophenotyping protocols for CD4<sup>+</sup> T-cell enumeration have been designed, with the purpose to select cost-effective protocols for any given existing instrument. This section covers only those candidates for “the affordable CD4 T-cell enumeration method,” that are developed for existing flow cytometers for clinical use. In general, protocols with fewer reagents are more cost effective. A protocol called “PanLeucogating” was introduced as both a double- and a single-platform technology in South Africa,<sup>1</sup> and was successfully applied in subsequent independent studies.<sup>2,3,4</sup> Both technologies work with a lineage marker-assisted gating strategy requiring only two mAb (i.e., CD45 and CD4).

### B2. Three-Color “T-gating” Protocol

The first lineage marker-assisted protocol for CD4<sup>+</sup> T-cell enumerating was introduced in 1992.<sup>5</sup> This approach takes advantage of the consistent surface CD3 receptor expression by all functionally mature T-cells. With the T-gating protocol, two histograms are generated as seen in Figure B1. The first histogram uses CD3 fluorescence (FL) and sideward light scatter (SSC). T-cells are identified inside the broken red square. In the second histogram, CD3 T-cells are selected by a dual fluorescence protocol analyzing for CD4 and CD8 receptor. The T-gating protocol was originally developed to provide T-cell subsets as the percentage of total T-cells.<sup>5</sup> One of the unique features of this simple gating strategy is that it has its own built-in free isotype control (see Figure B2). However, this novel approach was not widely accepted for quite some time, and the protocol was not implemented in commercially available assay kits until 1995. This system, which was dedicated to CD4/CD8 T-cell enumeration, implemented T-gating as a two-color protocol. This protocol uses two tubes with dual-stained samples, one tube for CD3 vs. CD4 and the other for CD3 vs. CD8. The original software combined the CD3 values from the two assays as part of an internal QC feature. More recently, these two assays can be purchased separately, thus the software was changed. In addition, each tube contains a predefined amount of counting microfluorospheres. As most instruments for routine clinical use have three- and four-color capacity, the most popular version of the T-gating protocol is illustrated in Figure B1. In Figure B1, the first histogram is generated with SSC on the vertical axis and CD3 fluorescence on the horizontal axis. This combination permits a well-resolved separation between CD4 T-cells and monocytes. In addition, this analytical approach provides good discrimination between all other cell populations, such as the granulocytes (G) and B-cells (B). The second histogram is generated exclusively from “T-gated,” CD3<sup>+</sup> events, with CD4 and CD8 set out on the horizontal and vertical axes, respectively. This format provides an opportunity to identify T-cells that are neither CD4<sup>+</sup> or CD8<sup>+</sup>, nor expressing both markers.

## Appendix B. (Continued)

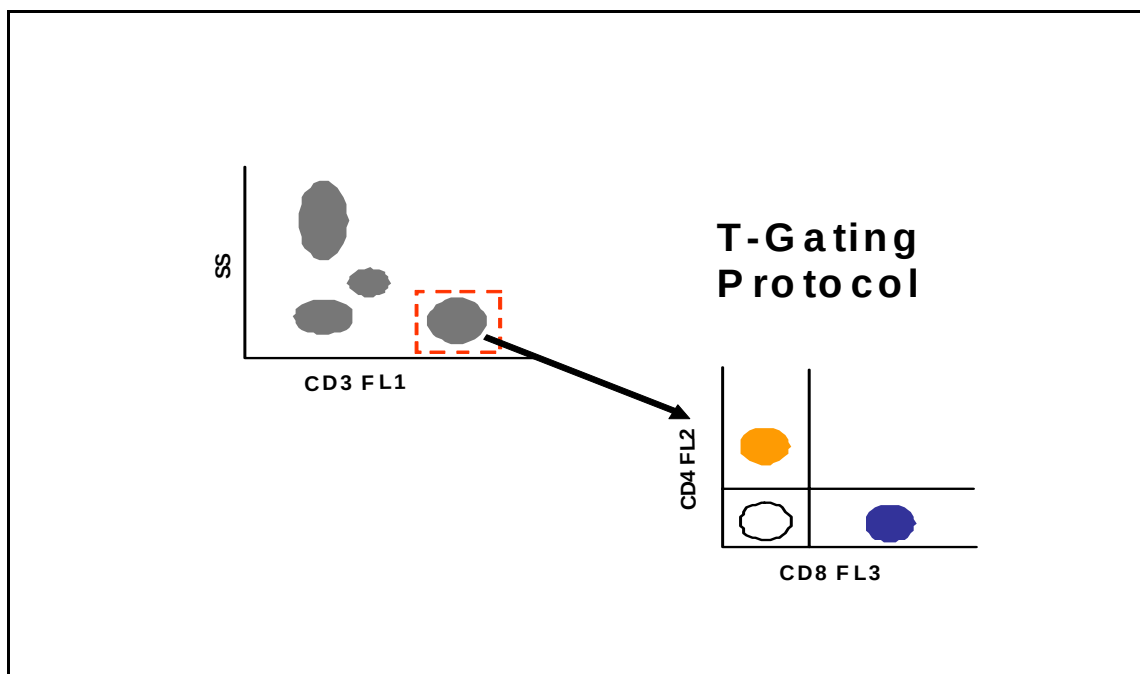


**Figure B1**

The T-gating approach also offers an internal negative control for CD3<sup>+</sup> T-cells. The background staining can be observed as all the WBC undergo some nonspecific staining with CD3 mAb (see Figure B2). All the cells that are inside the blue area (large oval) have some nonspecific CD3 reagents attached to them, as they are not immediately along the Y axis. Note the space occupied by the two black arrows (see Figure B2). Cells in the green circle (circle in large oval) represent the B-cells population that serves as the true isotype control for the T-cells.

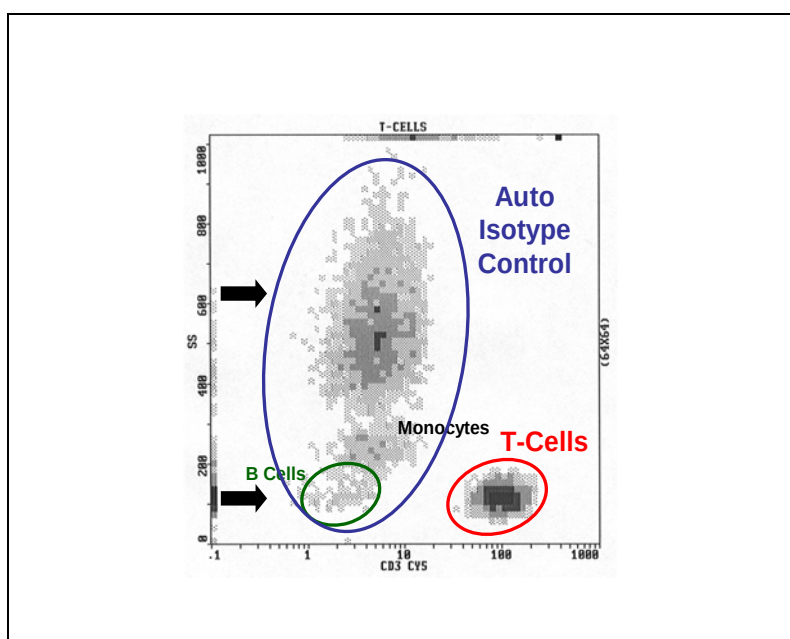


**Figure B2**

## Appendix B. (Continued)

### B3. Two-Color “PanLeucogating” Protocol

The original PanLeucogating protocol, described by Glencross et al,<sup>1</sup> uses only 2 mAb: CD45 and CD4. In this CD45-assisted gating method, a large gate is drawn around all CD45<sup>+</sup> WBC on a plot of CD45 FITC vs. SSC: the PanLeucogate (see Figure B3, histogram 1.1, region A). This gate provides the denominator (i.e., all WBC) for subsequent absolute count calculations. On a second plot of CD4PE vs. SSC, a gate is drawn around the CD4<sup>++</sup> (bright) lymphocytes with low side scatter (see Figure B3, histogram 1.2, region B). The absolute CD4 T-cell number is calculated from:

$$(\text{number of events in Gate B} / \text{number of events in Gate A}) \times \text{WBC count} (\times 10^9/\text{L})$$

The CD4<sup>+</sup> T-cell fraction of all WBC (Gate B/Gate A) is obtained from a flow cytometer, and the absolute CD4<sup>+</sup> T-cell count is calculated using a simultaneous WBC count from a hematology analyzer. The innovative feature of this protocol is the elimination of the absolute lymphocyte count determination from a hematology analyzer. This single change in the protocol can reduce the inherent variability of the dual-platform results currently generated with some predicate methods used in resource-rich countries.

The percent CD4<sup>+</sup> T-cells (expressed as proportion of lymphocytes), a parameter used for monitoring HIV-infected children, is calculated from:

$$(\text{number of events in Gate B} / \text{number of events in Gate C}) \times 100\%$$

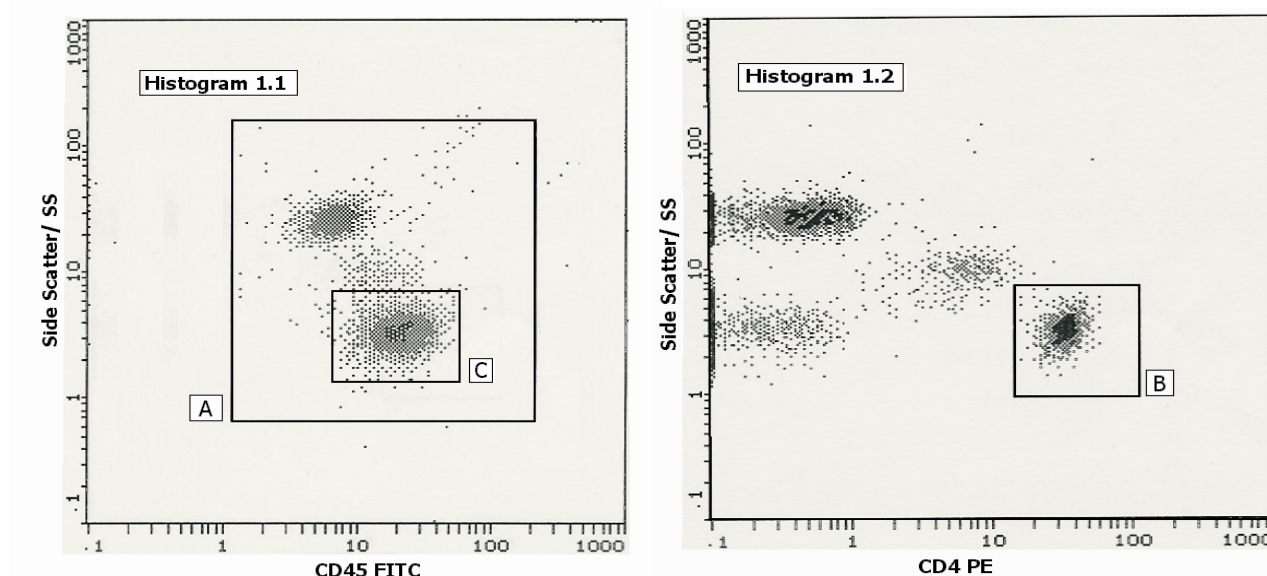


**Figure B3. The Two-Color “PanLeucogating” Protocol.** For explanation, see text above.

## References for Appendix B

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## **Appendix C. Alternative Technologies for CD4<sup>+</sup> T-cell Enumeration**

### **C1. Background**

In 2003, the World Health Organization (WHO) introduced an initiative called “3 by 5.” It was a global commitment to treat 3 000 000 people living with HIV/AIDS by the end of 2005. This initiative did not reach the target number in 2005, but it did accelerate access to effective treatment. This ambitious plan to scale up antiretroviral therapy in resource-limited settings relies on clinical disease staging that includes enumeration of CD4<sup>+</sup> T-cells. Therefore, early in the 21<sup>st</sup> century, biomedical platforms such as those made for enumerating T-cell subsets needed to be re-engineered to bring down assay costs in resource-poor regions. T-cell subset assay price must be reduced dramatically to match the diminishing cost of antiretroviral therapy. By 2004, WHO recognized that it is unlikely that global implementation of antiretroviral therapy will be realized with the execution of the existing costly methods.<sup>1,2</sup> To expedite the generation of scientific evidence for inclusion of some of the recently emerging alternative technologies, WHO introduced a universal multisite evaluation protocol. New technologies, not yet approved by major agencies that comply with the WHO evaluation requirements, will be considered for global use by WHO and all of its affiliated agencies. In this section, some of the alternative technologies that may eventually pass the rigor of the WHO evaluation are briefly discussed.

### **C2. A Classification System for Alternative Flow Cytometric Approaches**

This classification strategy reflects some of the innovative technical features that are available on some recently introduced flow cytometers. It also looks at applications of alternative gating protocols as they are implemented on some of the “more affordable” clinical instruments. There are also several additional methods that are not based on FCM. These methods range from traditional manual microscopy to novel automated digital image capture-based instrumentation. There are some cell-counting methods that do not use an optical flow chamber. Such static cell-counting methods are beyond the scope of this document.

### **C3. Instrument Classification Based on Technical Features Available on Some of the Novel Flow Cytometers**

Until recently, the source of cell propulsion through the flow chamber was always accomplished with the assistance of sheath fluid. It is obvious that an instrument that does not require sheath fluid (i.e., filtered saline) will be the preferred CD4 T-cell counting technology in regions such as sub-Saharan Africa. In one of the recently introduced alternative instruments, the traditional hydrodynamic focusing was replaced with microcapillary fluidics that eliminates the need for sheath fluid. In another instance, the old paradigm of fluorescently labeled detection strategy was challenged. The introduction of a new tagging option instigated a reclassification of methods based on the type of label used for tagging mAb for immunophenotyping. In the case of another recently developed technology, the traditional fluorescence label is replaced with a CD4 mAb that is tagged with colloidal gold. The detection of this nonfluorescent tag is accomplished with a wide-angle light scatter detector.

### **C4. Protocol Classification Based on Alternative Gating Strategies**

For 20 years, the gating paradigm to identify clusters of leukocyte subsets was based on the use of forward (FSC) and sideward light scattering (SSC). The use of these intrinsic cell properties is no longer endorsed by most guidelines written by the major agencies.<sup>1,2</sup> Currently accepted methodologies use a combination of extrinsic and intrinsic cell attributes, such as bright CD45 expression and SSC, respectively. Some of the alternative protocols embrace leukocyte subset cluster identification based on lineage or function-specific markers. Electronic gates are generated around tagged cells based on extrinsic

## Appendix C. (Continued)

cell features with or without an intrinsic cellular parameter. The most frequently used option is the lineage-specific leukocyte cluster gating, combined with the SSC parameter. Of these “mixed” gating strategies, the one combining CD45 bright fluorescence with SSC gating is the most popular.<sup>3</sup> There are two dedicated flow cytometer instruments that use the T-gating (CD3) option and another two use a leukocyte function-specific marker alone (i.e., the CD4 molecule). This type of direct gating strategies is referred to as primary gating. One instrument uses the CD4 marker alone, and the other instrument integrates it in combination with SSC for CD4<sup>+</sup> T-cell enumeration.<sup>4</sup> In the case of the first instrument, a single-parameter histogram is generated with the aid of two manually set cursors to delineate the bright CD4<sup>+</sup> T-cells from the dim CD4<sup>+</sup> monocytes. In the case of the second instrument, a manual gate is drawn around the bright CD4 events to exclude the monocytes.

The following are examples of methodologies available on dedicated instruments for CD4 T-cell enumeration:

- microcapillary FCM
- functional marker-based gating (CD4)
- nonfluorescence FCM with autogating (CD4)
- lineage-specific autogating (CD3)
- lineage-specific gating (CD3)
- functional marker-based gating (CD4)

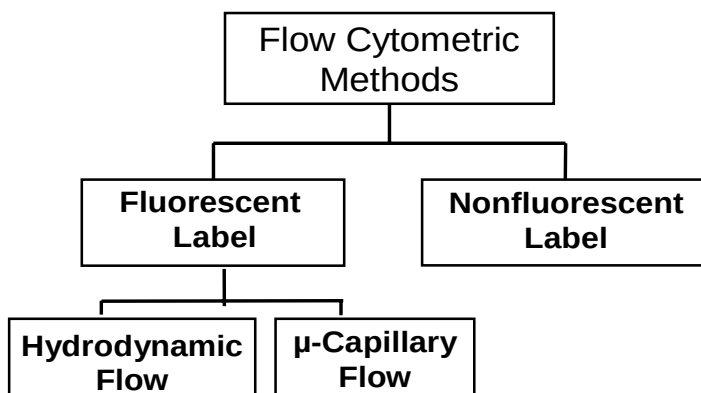


**Figure C1. A Schematic Overview of Alternative CD4<sup>+</sup> T-cell Counting Technologies**

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## **Appendix D. Enumeration of Immunologically Defined Rare Cell Populations**

### **D1. Introduction**

A careful analysis of the assay requirements is critical for the development of strategies for detection and/or isolation of rare cells of interest. Does the assay require accurate frequency information? If so, FCM is probably the best technique to employ. Today's bench-top flow cytometers are able to reliably detect rare cells at frequencies as low as 1 in  $10^5$ - $10^6$  by combining multicolor staining and rigorous cleaning of the flow system.<sup>1-5</sup> For detection of cells at frequencies of 1 in  $10^7$  or lower, enrichment and purification steps will be needed to overcome a fundamental flow cytometric problem, the relatively limited rate at which cells can be evaluated. When enumerating cells at a speed of 10 000 cells/sec, which is available only on the most recently developed bench-top flow cytometers, it will take a minimum of one hour to count  $36 \times 10^6$  cells, which would only be capable of detecting  $\sim 4$  cells in  $10^7$ .<sup>1,6</sup> Another limitation is posed by the amount of sample available for rare cell detection. For example, to detect five positive cells within  $10^7$  WBC in a blood sample of a healthy donor (with  $\sim 5 \times 10^9$  WBC/L), a theoretical minimum of 10 mL of blood would require processing, and in reality much more.

If a detection threshold lower than 1 in  $10^6$  cells is needed, one or more pre-enrichment procedures will be needed. Such procedures may consist of density gradients (e.g., Percoll or Ficoll-Hypaque), immunomagnetic sorting, affinity columns (with attached ligands or antibodies), sedimentation (e.g., unit gravity sedimentation, velocity sedimentation), or high-speed cell sorting before FCM analysis will be necessary. The result of each enrichment procedure can be expressed in terms of recovery (i.e., yield) and purity of the cells of interest.<sup>1,4</sup> The section below focuses on FCM for rare event detection, as this technique is the core of this section.

### **D2. Recommendations for FCM-Based Assays to Detect Rare Events**

#### **D2.1 Multicolor and Sequential Gating Strategies**

Multicolor staining in combination with sequential gating strategies were proven as useful tools for the identification of rare cells. (See also Part C of this document.)

#### **D2.2 Exclusion of Nonspecific Binding**

For accurate detection of rare events, background signals ("noise") must be substantially less than the frequency of the events sought. Thus, for rare cells at a frequency of 0.01% of total cells, the cytometric assay would need to be greater than 99.99% free of noise. Background noise is generally due to a variety of factors that yield fluorescent signals, or at least signals perceived by the cytometer to be fluorescent, in the absence of antibodies or fluorescent probes specific to the cells of interest. Several layers of controls are necessary to evaluate background and establish the veracity of specific staining in rare event analysis. As in the assays described above (i.e., enumeration of lymphocyte subsets and CD34<sup>+</sup> hematopoietic stem cells), fluorochrome-matched isotype controls are considered less relevant. An isotype control antibody will never be entirely representative for the particular antibody for which it should control. Even with freshly drawn blood, it is not unusual to observe nonspecific binding of isotype controls representing from 0.1 to 0.5% of cells analyzed. While this level of staining does not significantly influence results for routine clinical samples, it greatly affects the outcome of rare event analysis. Binding of isotype controls to cells may be the result of dead or dying cells, cells bearing Fc receptors, and several other factors. Alternative methods to control for nonspecific binding include the following: (1) inclusion of a real-time viability stain (such as 7-aminoactinomycin D [7-AAD]) in each tube to exclude dead cells; (2) whole blood staining or preincubation of the cells with a heat-inactivated blocking serum from the same

## Appendix D. (Continued)

species used to create the monoclonal antibodies to prevent Fc receptor-mediated binding; and (3) use of a dump channel to exclude from analysis cells of known immunophenotypes that are not of interest. The dump channel is one of the fluorescent channels (FL1, FL2, etc.) that is set aside for exclusion gating, and may contain more than one parameter or marker for exclusion. For example, for the analysis of nonleukocyte tumor cells, a dump channel may include markers for dead cells (7-AAD) and for WBC (CD45, CD3, CD19, and CD33). Finally, as completely unstained cells have very different levels of background than cells that have been stained with multiple reagents, the optimal control for any marker of interest in a multicolor staining is a staining that contains all reagents except the one of interest (a.k.a. “fluorescence minus one”).<sup>7,8</sup> This approach allows a rigorous application of positive vs. negative selection of the events of interest.

### D2.3 Minimal Number of Events of Interest to Acquire

In rare event analysis, large numbers of cells must be counted to obtain statistically meaningful numbers of rare cells.<sup>1,6</sup> Rare events typically follow a Poisson distribution, according to which their CV (in %) is  $100 \times \sqrt{n} \div n$ , in which  $n$  = number of events fulfilling the flow cytometric criteria of the rare events under study. Hence, their CV will vary proportionately as the square root of the number of rare events acquired. This contention was confirmed by triplicate assessments of percent CD34<sup>+</sup> cells, in which the CV was highly dependent on the percent CD34<sup>+</sup> cells in the specimen if a fixed number of nucleated cells (i.e., 50 000) was counted, and fairly constant (~6.5%) upon counting of a fixed number (i.e., 250) of CD34<sup>+</sup> cells.<sup>9</sup> Thus, the acquisition of a minimum of 100 rare events ensures a CV of 10%, that generally is considered acceptable. When >1 000 000 or more cells must be analyzed, huge list-mode data files will be created, whose precise sizes are also determined by the number of parameters collected, the resolution used, and the number of cells collected. Although file sizes are not nearly as limited by computing capability as in earlier years, enormous files can be problematic for some computers and software programs. To decrease file size and increase ease of file management, events are often collected so that only the events of interest are saved (i.e., live gating).

### D2.4 Prevention of Carry-over

Another source of background in rare event analysis is carry-over of cells in the cytometer from previous experiments. Before rare event analysis on any cytometer, extensive cleaning and washing is recommended to remove residual cells and particles. The extent and nature of this cleaning will differ with the make and model of cytometer.

### D2.5 Doublet Discrimination

Use doublet discrimination options to detect cell aggregates and coincidence of both rare and total events, to eliminate their influence on the frequency.

## References for Appendix D

- <sup>1</sup> Shapiro HM. *Practical Flow Cytometry*. 4th ed. New York: Wiley-Liss; 2003.
- <sup>2</sup> Ryan DH, Mitchell SJ, Hennessy LA, Bauer KD, Horan PK, Cohen HJ. Improved detection of rare CALLA-positive cells in peripheral blood using multiparameter flow cytometry. *J Immunol Methods*. 1984;74(1):115-128.
- <sup>3</sup> Gross HJ, Verwer B, Houck D, Recktenwald D. Detection of rare cells at a frequency of one per million by flow cytometry. *Cytometry*. 1993;14(5):519-526.
- <sup>4</sup> Leary JF. Strategies for rare cell detection and isolation. *Methods Cell Biol*. 1994;42:331-358.
- <sup>5</sup> Gross HJ, Verwer B, Houck D, Hoffman RA, Recktenwald D. Model study detecting breast cancer cells in peripheral blood mononuclear cells at frequencies as low as 10<sup>-7</sup>. *Proc Natl Acad Sci USA*. 1995;92(2):537-541.
- <sup>6</sup> Rosenblatt JI, Hokanson JA, McLaughlin SR, Leary JF. Theoretical basis for sampling statistics useful for detecting and isolating rare cells using flow cytometry and cell sorting. *Cytometry*. 1997;27(3):233-238.
- <sup>7</sup> Baumgarth N, Roederer M. A practical approach to multicolor flow cytometry for immunophenotyping. *J Immunol Meth*. 2000; 243L 77-97.
- <sup>8</sup> Roederer M. Spectral compensation for flow cytometry: visualization artifacts, limitations, and caveats. *Cytometry*. 2001;45:194-205.
- <sup>9</sup> Serke S, Arseniev L, Watts M, et al. Imprecision of counting CFU-GM colonies and CD34-expressing cells. *Bone Marrow Transplant*. 1997;20:57-61.

**Clinical and Laboratory Standards Institute consensus procedures include an appeals process that is described in detail in Section 8 of the Administrative Procedures. For further information, contact CLSI or visit our website at [www.clsi.org](http://www.clsi.org).**

## Summary of Comments and Working Group Responses

H42-A2: *Enumeration of Immunologically Defined Cell Populations by Flow Cytometry; Approved Guideline—Second Edition*

### General

1. I think this guideline is an invaluable tool for characterizing hematolymphoid neoplasia and an appropriate addition to the previous version of H42, which basically did not address this particular issue. The emphasis on sample quality control using morphologic correlation while interpreting flow results is greatly appreciated and cannot be over-emphasized.
  - **No modification is needed in response.**
2. I would like to vote in favor of this document. Although long and detailed, it very nicely integrates enumeration of lymphocyte subsets and enumeration of CD34+ stem cells by flow cytometry. I believe that the quality control guidelines outlined in this document will help to reduce interlaboratory discrepancies, a potential problem we currently occasionally come across.
  - **No modification is needed in response.**
3. As requested, we have reviewed the attached CLSI document. In theory, the requirements of this standard are very good and very thorough. The layout and descriptions and ease of understanding are acceptable as the completeness of directions.
  - **No modification is needed in response.**
4. It should be noted that the recommendations are very labor intensive and wouldn't work for most labs, making this document ineffective.
  - **H42 contains recommendations that are meant to serve as a basis for a laboratory protocol. From there, formats accommodating efficient daily working practice can be developed (e.g., such as provided in *Current Protocols for Cytometry*), adjusted to the user's specific needs.**

### Foreword

5. We echo the sentiments in the Foreword on page vii (4th paragraph): "The method as outlined is laborious and time-consuming. In its complete form, it may not be acceptable to many laboratories."

Our recommendation would be to produce a more realistic, useable version.

- **See the response to Comment 4.**

### Section 5.1, Definitions

6. After reviewing the 60 plus-page H42-A2 guideline, I found it interesting and useful, the references are up to date, and it is a good guide. I did find the definition of CD (page 4) interesting; it is written as "cluster differentiation." The convention that I am most familiar with defines it as: cluster of differentiation. The lack of a preposition certainly does not doom the document; however, it should be included in the definition.
  - **The text has been revised as suggested.**

Section 6.8, Blood Spills

7. The statement does not indicate that bleach must be prepared daily. You could also refer the reader to safety documents for additional information.
- **The text has been revised as suggested and CLSI/NCCLS document GP17—*Clinical Laboratory Safety* has been referenced at the end of this paragraph.**

Section 7.2, Dual-Platform (DP) Methods

8. The last line in the paragraph refers to SP methods in Section 7.4. SP methods start at Section 7.3.
- **The text has been revised to refer to Section 7.3 for single-platform methods.**

Section 7.5.2.2, Verification of Pipetting Accuracy

9. It is not clear that the information and example all relate to a 100- $\mu$ L pipette.
- **The sentence “Here, a 100- $\mu$ L pipette is used as an example” has been added to Section 7.5.2.2.**

Section 10.5, Temperature Extremes

10. The touch method to check temperature upon arrival would not be sufficient. Many labs now include a sensor to detect a maximum/minimum indicator in the package to ensure extreme temperatures were not reached and then adjusted before arrival.
- **This paragraph has been rephrased as follows:**

**“If the specimen tube has been transported to the laboratory from outside the institution, it may have been exposed to temperature extremes; however, examination upon receipt to determine if it is unusually warm or cold to the touch is not sufficient. For all deliveries, except those on the same day, it is recommended to include a minimum-maximum temperature sensor in the package to document whether or not extreme temperatures have been reached during shipment.”**

**NOTES**

## The Quality Management System Approach

Clinical and Laboratory Standards Institute (CLSI) subscribes to a quality management system approach in the development of standards and guidelines, which facilitates project management; defines a document structure via a template; and provides a process to identify needed documents. The approach is based on the model presented in the most current edition of CLSI/NCCLS document HS1—*A Quality Management System Model for Health Care*. The quality management system approach applies a core set of “quality system essentials” (QSEs), basic to any organization, to all operations in any healthcare service’s path of workflow (i.e., operational aspects that define how a particular product or service is provided). The QSEs provide the framework for delivery of any type of product or service, serving as a manager’s guide. The quality system essentials (QSEs) are:

|                     |                        |                                   |                     |
|---------------------|------------------------|-----------------------------------|---------------------|
| Documents & Records | Equipment              | Information Management            | Process Improvement |
| Organization        | Purchasing & Inventory | Occurrence Management             | Customer Service    |
| Personnel           | Process Control        | Assessments—External and Internal | Facilities & Safety |

H42-A2 addresses the quality system essentials (QSEs) indicated by an “X.” For a description of the other documents listed in the grid, please refer to the Related CLSI/NCCLS Publications section on the following page.

| Documents & Records | Organization | Personnel | Equipment          | Purchasing & Inventory | Process Control                                      | Information Management | Occurrence Management | Assessments—External and Internal | Process Improvement | Customer Service | Facilities & Safety      |
|---------------------|--------------|-----------|--------------------|------------------------|------------------------------------------------------|------------------------|-----------------------|-----------------------------------|---------------------|------------------|--------------------------|
|                     |              |           | X<br>H43<br>I/LA24 | H3                     | X<br>C28<br>GP5<br>H3<br>H20<br>H43<br>I/LA24<br>M29 |                        |                       | I/LA24                            | I/LA24              |                  | GP5<br>GP17<br>H3<br>M29 |

Adapted from CLSI/NCCLS document HS1—*A Quality Management System Model for Health Care*.

### Path of Workflow

A path of workflow is the description of the necessary steps to deliver the particular product or service that the organization or entity provides. For example, CLSI/NCCLS document GP26—*Application of a Quality Management System Model for Laboratory Services* defines a clinical laboratory path of workflow which consists of three sequential processes: preexamination, examination, and postexamination. All clinical laboratories follow these processes to deliver the laboratory’s services, namely quality laboratory information.

H42-A2 addresses the clinical laboratory path of workflow steps indicated by an “X.” For a description of the other documents listed in the grid, please refer to the Related CLSI/NCCLS Publications section on the following page.

| Preexamination       |                   |                  |                           | Examination     |                              |                | Postexamination                 |                   |
|----------------------|-------------------|------------------|---------------------------|-----------------|------------------------------|----------------|---------------------------------|-------------------|
| Examination ordering | Sample collection | Sample transport | Sample receipt/processing | Examination     | Results review and follow-up | Interpretation | Results reporting and archiving | Sample management |
| X<br>H3<br>H43       | X<br>H3<br>H43    | X<br>H3<br>H43   | X<br>H3<br>H20<br>H43     | X<br>H20<br>H43 | X<br>H20<br>H43              | X<br>H43       | X<br>H43                        |                   |

Adapted from CLSI/NCCLS document HS1—*A Quality Management System Model for Health Care*.

## Related CLSI/NCCLS Publications\*

- C28-A2**      **How to Define and Determine Reference Intervals in the Clinical Laboratory; Approved Guideline—Second Edition (2000).** This document contains guidelines for determining reference values and reference intervals for quantitative clinical laboratory tests.
- GP5-A2**      **Clinical Laboratory Waste Management; Approved Guideline—Second Edition (2002).** Based on US regulations, this document provides guidance on the safe handling and disposal of chemical, infectious, radioactive, and multihazardous wastes generated in the clinical laboratory. While a valuable resource for a wider audience, it is intended for use primarily in the United States.
- GP17-A2**     **Clinical Laboratory Safety; Approved Guideline—Second Edition (2004).** This document contains general recommendations for implementing a high-quality laboratory safety program, which are provided in a framework that is adaptable within any laboratory.
- H3-A5**        **Procedures for the Collection of Diagnostic Blood Specimens by Venipuncture; Approved Standard—Fifth Edition (2003).** This document provides procedures for the collection of diagnostic specimens by venipuncture, including line draws, blood culture collection, and venipuncture in children. It also includes recommendations on order of draw.
- H20-A2**      **Reference Leukocyte (WBC) Differential Count (Proportional) and Evaluation of Instrumental Methods; Approved Standard—Second Edition (2007).** Discusses automated differential counters and establishes a reference method based on the visual (or manual) differential count for leukocyte differential counting to which an automated or manual test method can be compared, and an experiment to carry out the comparison. Describes procedures for collecting specimens; preparing blood films and requirements for acceptable wedge and spun films; Romanowsky staining; the formed elements; variant leukocyte forms; and a protocol for examining blood films. Details procedures for determining inaccuracy, and within-run and between-run imprecision; procedures for determining sensitivity/specificity/predictive values of flags; and statistical methods for determining inaccuracy and imprecision.
- H43-A2**      **Clinical Flow Cytometric Analysis of Neoplastic Hematolymphoid Cells; Approved Guideline—Second Edition (2007).** This document provides performance guidelines for the immunophenotypic analysis of neoplastic hematolymphoid cells using immunofluorescence-based flow cytometry; for sample and instrument quality control; and precautions for acquisition of data from neoplastic hematolymphoid cells.
- I/LA24-A**     **Fluorescence Calibration and Quantitative Measurement of Fluorescence Intensity; Approved Guideline (2004).** This guideline describes the basic principles, reference materials, and laboratory procedures upon which quantitative fluorescence calibration is based.
- M29-A3**      **Protection of Laboratory Workers From Occupationally Acquired Infections; Approved Guideline—Third Edition (2005).** Based on US regulations, this document provides guidance on the risk of transmission of infectious agents by aerosols, droplets, blood, and body substances in a laboratory setting; specific precautions for preventing the laboratory transmission of microbial infection from laboratory instruments and materials; and recommendations for the management of exposure to infectious agents.

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\* Proposed-level documents are being advanced through the Clinical and Laboratory Standards Institute consensus process; therefore, readers should refer to the most current editions.

**NOTES**

**NOTES**

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|                                                                                     |                                                               |                                                                   |                                                                                   |
|-------------------------------------------------------------------------------------|---------------------------------------------------------------|-------------------------------------------------------------------|-----------------------------------------------------------------------------------|
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| Dr. Everette Chalmers Regional<br>Hospital (NB)                                     | Kaiser Permanente (CA)                                        | Oklahoma Heart Hospital, LLC (OK)                                 | Shands at the University of Florida                                               |
| Driscoll Children's Hospital (TX)                                                   | Kaiser Permanente (MD)                                        | Onze Lieve Vrouw Ziekenhuis<br>(Belgium)                          | Shape Healthcare Clinic (APO, AE)                                                 |
| DSI of Bucks County (PA)                                                            | Kangnam St. Mary's Hospital<br>(Korea)                        | Orlando Regional Healthcare System<br>(FL)                        | Sheik Kalifa Medical City (UAE)                                                   |
| DUHS Clinical Laboratories (NC)                                                     | Kenora-Rainy River Reg. Lab.<br>Program (Canada)              | Our Lady of the Way Hospital (KY)                                 | Shore Memorial Hospital (NJ)                                                      |
| Dumfries and Galloway Royal<br>Infirmary (Scotland)                                 | King Fahad Medical City (Saudi<br>Arabia)                     | Overlook Hospital (NJ)                                            | SJRMCM Plymouth Laboratory (IN)                                                   |
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| Fleury S.A. (Brazil)                                                                | LifeBridge Health Sinai Hospital (MD)                         | Pitt County Memorial Hospital (NC)                                | State of Washington Public Health Labs                                            |
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